THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. VII OCTOBER, 1926 No. 5

THE ULTRA-MICROSCOPIC VIRUSES: I.—CELL INCLUSIONS ASSOCIATED WITH CERTAIN ULTRA-MICROSCOPIC DISEASES—A PICTOGRAPHIC REVIEW.

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Received for publication July 22nd, 1926.

INTRODUCTION.

THE importance of all problems connected with ultra-microscopic viruses has prompted us to review in pictographic form the voluminous literature already existing on the intracellular inclusions associated with certain diseases due to ultra-microscopic viruses.

It has been necessary to exclude from this review numerous figures which lack clear cytological detail or which reproduce facts already well known. Reference, however, will be found in the bibliography to all the more important papers on the subject.

On their discovery the greater number of cell inclusions were named after their discoverers—Guarnieri's bodies in variola and vaccinia, Mallory's bodies in scarlet fever, Negri's bodies in rabies. At first these bodies were regarded by many investigators as protozoa with elaborate life-cycles. This view has now been discarded, though many still believe in the compromise suggested by von Prowazek (1907), according to which the inclusion bodies are of a dual nature, consisting of micro-organisms embedded in material deposited around them as the result of a reaction of the cell-protoplasm. The term "Chlamydozoa"—literally cloak animals—was proposed as a name for this class of organism. Lipschütz (1912) devised the term "Strongyloplasmata" to include the same group of organisms, while later (1921) he suggested a classification into three groups:

(1) Cyto-oikon group in which the enclosures lie in the cytoplasm—molluscum contagiosum, trachoma, fowl-pox, sheep-pox.

(2) Karyo-oikon group in which the enclosures lie in the nucleus—herpes, warts.

(3) Cyto-karyo-oikon group in which the enclosures lie in the cyto- and nucleoplasm—variola and para-vaccinia.

It is doubtful whether in the present state of our knowledge the term "Chlamydozoa" can be justified as a cloak for anything except our ignorance, while any attempt at classification of the ultra-microscopic viruses is premature.

The following diseases are discussed in this review:

Variola and vaccinia.
Sheep-pox.
Fowl-pox.
Scarlet fever.
Molluscum contagiosum.
Trachoma.
Rabies.
Distemper.
Varicella.
Herpes.
Meningo-encephalo-myelitis of horses.
Virus III infection of rabbits.
Paravaccinia.
Infections of unknown ætiology.
Virus from guinea-pigs.
Foot and mouth disease.
Warts.
Polyhedral and other ultravirus diseases of insects.
Fowl plague.
Swine fever.
African horse sickness.
A disease of fowls.

It is probable that as more refined cytological methods are employed other ultra-microscopic virus diseases will be found to be associated with specific changes within the cell. The great uncertainty now existing as to the nature and origin of cell inclusions is apparent from an examination of the figures.

(1) *Variola and Vaccinia.*

Although Renaut (1881) described spherical globules within the epidermal cells of variolous lesions, his communication was unaccompanied by figures. Guarnieri (1892) was thus the first to figure the intracellular bodies which now bear his name. He regarded them as protozoa, and gave them the name of "*Cytoryctes vaccinæ*" (Fig. 1). Complicated life-cycles were described by Councilman, Magrath and Brinckerhoff (1904), Calkins (1904), and von Prowazek (1912). Other observers such as Copeman and Mann (1898–9) (Fig. 2), and Ewing (1904–5) (Figs. 3 and 4), brought forward evidence to show that vaccine bodies arise from some preformed cell constituent. The latter in particular believed that they were caused by an outpouring and precipitation of nuclear protein in the cytoplasm. Conflicting views have been expressed as to the nuclear, or cytoplasmic origin of vaccine bodies. Some observers, such as Böing (1920) (Fig. 5), believe that the vaccine virus itself may be demonstrated in the cytoplasm, and more rarely in the nucleus, as minute granules which stain a deep red with Azur I. Recently, Woodcock (1921) has revived the old idea that vaccine bodies arise through the digestion of red blood-cells. Cowdry (1922), however, points out that no trace of hæmoglobin coloration can be found in vaccinated corneal cells observed in the fresh state. The theory that vaccine bodies are formed through the phagocytosis of leucocytic fragments is also untenable. Cowdry (1922) (Figs. 6 and 7), in a detailed study, shows that vaccine bodies arise from a substance normally present in the cell in small amounts. Under the stimulus of vaccination this material rapidly increases in amount, and shows evidence of heterogeneity both in living and fixed tissue preparations. Thus supravital staining of vaccine bodies reveals the presence of blue staining droplets, or

granules, within larger pink staining masses. Some vaccine bodies contain particles of high refractive index and irregular shape which do not stain. Occasionally these particles occur free in the cytoplasm. As the larger vaccine bodies are formed they lose their homogeneity as the result of either dissociation, or the taking up of other substances from the cytoplasm. There is no evidence that vaccine bodies contain any micro-organisms demonstrable by ordinary optical means, though if the virus is beyond the limit of visibility of the best lenses its relation to the vaccine bodies is not easily determined.

In a more recent paper, Schütz (1925; Fig. 8) expresses the opinion that Guarnieri bodies are neither amœba-like nor of the nature of Chlamydozoa: they do not originate from extruded nucleoli or degenerate cell organs, but are true cytoplasmic structures arising as the result of infection of the cell by the vaccinia virus.

(2) *Sheep-pox.*

Although a number of experimental studies have shown that the viruses of vaccinia, sheep, horse, swine and goat-pox are closely related if not identical, there have been comparatively few observations on the cytology of pox in domestic animals other than the calf. Such observations as have been made show that Guarnieri-like bodies are present in the infected epidermal cells (Borrel, 1903; Fig. 9).

(3) *Fowl-pox.*

Bollinger (1873) seems to have been one of the first to describe the large cytoplasmic inclusions which are found in the epidermal cells in this disease (Fig. 10; Apolant, 1903. Fig. 11; Michælis, 1903). In much of the earlier work on the subject these inclusions were regarded as parasites, but though this view has now been discarded the actual nature of the bodies is still obscure. They are partly of a lipoid character.

(4) *Scarlet Fever.*

The nature of the bodies described by Mallory (1904) (Fig. 12) in the epidermal cells in scarlet fever also remains obscure, since few observers have been able to confirm his observations.

Döhle (1912) has described oval or slightly elongated bodies closely resembling cocci in the protoplasm of polymorphonuclear leucocytes in this disease. Similar bodies are found in most diseases caused by pyogenic organisms. They are probably products of cytoplasmic degeneration.

(5) *Molluscum Contagiosum.*

Little need here be said in regard to the nature of the so-called "molluscum bodies." Though their parasitic nature cannot now be maintained, opinion as to their origin has scarcely advanced since MacCallum (1892) (Fig. 13)

suggested that they were formed as the result of nucleolar extrusion. Nucleolar extrusion, however, is a phenomenon associated with normal keratinization in most epidermal cells.

(6) *Trachoma.*

The cytoplasmic inclusions found in the epithelial cells of the conjunctiva in this condition were first described by Halberstädter and v. Prowazek (1907). When stained by Giemsa's method they are seen (Noguchi and Cohen, 1913; Fig. 14) as small round or ovoid granules of a reddish-violet colour, sometimes surrounded by a clear halo—the initial bodies. Later, as the number of these corpuscles increases, they become surrounded by a blue staining substance which has been named plastin. Solovief (1921) (Fig. 15) has described small round or oval blue-staining masses in certain of the epithelial cells of the conjunctiva; these little corpuscles, which are often vacuolated, are not specific for trachoma. Often immediately external to them there is to be found a single red-staining granule. According to this observer trachoma bodies represent the products of nuclear degeneration. The significance of v. Prowazek's bodies has been further complicated by the fact that they have been frequently found in non-gonorrhœal conjunctivitis of the newborn. There are three possibilities in regard to non-gonorrhœal conjunctivitis of the newborn: (1) that it is a disease due to a virus allied to that of trachoma; (2) that the virus is the same in both diseases; (3) that trachoma bodies are due to the virus of non-gonorrhœal conjunctivitis of the newborn.

(7) *Rabies.*

Since the first description by Negri (1903) of the cytoplasmic inclusions found in the ganglion cells of the brain in rabies, an enormous number of papers have appeared either maintaining or denying the parasitic nature of these inclusions. Although practically no one now believes that these bodies are actually protozoa, there are many who are still of the opinion that the minute basophilic structures within the Negri bodies constitute the specific portion or true virus, while the eosin-staining portion is a form of capsule probably produced by the cell as a hyaline mantle. Thus Manouélian and Viala (1924) (Figs. 18 and 19) believe that the true parasite is a rod-shaped body which agglutinates and degenerates to form Negri bodies. They regard the "*Encephalitozoon rabiei*" as closely related to the "*Encephalitozoon cuniculi.*" Many observers, however, have been unable to demonstrate in Negri bodies "Innenkörper," some of which were uniformly hyaline, although in the same cell others might be seen with typical granular inclusions. Goodpasture (1925) (Figs. 20, 21 and 22) proposes the name "lyssa bodies" for those cell inclusions which have a homogeneous structure. He regards them as due to a transformation of the neurofibrillar material of the cell. Degenerative changes of mitochondrial filaments of axis-cylinders and of the cell-body are also found which form structures suggestive of early stages in the formation of both vacuolated Negri bodies and lyssa bodies. Acton and Harvey (1911) (Fig. 17) do not regard Negri bodies as entirely specific for rabies: they believe them to be associated with nucleolar extrusion.

(8) *Distemper.*

Lentz (1909) (Fig. 23), as the result of examination of the brains of dogs dying from distemper with nervous symptoms, described in the protoplasm of the cells of Ammon's horn small hyaline bodies rather smaller than a red blood-corpuscle. These inclusions stained deeply with eosin: they were sometimes intranuclear, occasionally extracellular. Standfuss (1908) confirmed these findings.

Sinigaglia (1912), however, reported small vacuolated bodies with basophilic granules in the brain-cells other than those of Ammon's horn, in the epithelial cells lining the ventricles, and in the epithelial cells of the cornea and bronchi. Babes and Starcovici (1912), while finding both the types of bodies described by Lentz and Sinigaglia, have also described in the cells of Ammon's horn granular inclusion bodies very similar to true Negri bodies except that the granules are more refringent. Kantorowicz and Lewy (1922–3) (Fig. 24) have described a parasite closely resembling *Encephalitozoon cuniculi* in the brains of dogs with nervous distemper. It seems probable that there is a form of encephalitis in dogs, at present confused with distemper, caused by an encephalitozoon parasite.

(9) *Varicella.*

Tyzzer (1906) (Fig. 25) was the first to describe inclusion bodies in the cells of varicella lesions in man. Nuclear and cytoplasmic inclusions were found both in epidermal cells and in the various cells of the corium. The nuclear inclusions measured 1–6μ in diameter; they stained red or purplish by the eosin-methylene blue method. The cytoplasmic inclusions rarely measured more than 4μ; they stained a deep purple, and in many a central granule was apparent. Inoculation of the cornea of the rabbit and skin of the monkey with varicella material gave negative results. Many other workers have tried without success to inoculate the rabbit's skin or cornea with varicella. Bertarelli (1909), however, claims to have produced varicella in the rabbit's skin. In the epidermal cells he found small inclusions which stained deeply with hæmatoxylin. These bodies appear to be of nucleolar origin.

Keysselitz and Mayer (1909) also described Chlamydozoa in the cytoplasm of human epidermal cells with varicellar lesions, while closely similar findings have been recorded by Swellengrebel (1911). Gins (1918) (Fig. 26) claims to have succeeded in inoculating the rabbit's skin with varicella. He figures specific cytoplasmic inclusions in the epidermal cells. Finally, Rivers (1926) (Fig. 27) has described intranuclear inclusions in the cells of the testicle of the Vervet monkey infected with human varicella material.

(10) *Herpes.*

Similar intranuclear bodies to those reported in varicella have been described in herpes by Lipschütz (1921) (Fig. 28), Luger and Lauda (1921), Lauda (1923) and Goodpasture (1925) (Fig. 35). The inclusions are associated with all forms of herpes in man and in the lesions produced experimentally in the rabbit by the herpetic virus.

The following staining reactions are given by Lipschütz (1921) as characteristic of herpetic intranuclear inclusions:

Stain.	Inclusions.	Nucleoli.
Giemsa	Red . . .	Dark blue.
Hæmatoxylin-eosin . .	Dark red . .	Blue black.
Heidenhain's iron-hæmatoxylin	Yellow grey .	Black.
Pappenheim . . .	Green or blue .	Red.

The inclusions can thus be distinguished by their tinctorial properties from chromatin and nucleoli, but in their earliest stages according to Goodpasture (1925) (Fig. 35) they are indistinguishable from material precipitated in the nucleoplasm by Helly's fluid.

Da Fano (1923) (Fig. 34), and Cowdry and Nicholson (1923) (Figs. 30, 31, 32, 33), in addition have described in experimental meningo-encephalitis in the rabbit various other granules, both nuclear and cytoplasmic, which are not specific for herpes and are dissimilar to those so clearly defined by Lipschütz.

Levaditi (1922) (Fig. 29) has produced in the rabbit what he believes to be an experimental lethargic encephalitis. In this condition he describes nuclear inclusions as "neurocorps encephalitiques." Doubt exists as to the true nature of Levaditi's encephalitis virus: in all probability it was a herpetic virus.

(11) *Meningo-encephalo-myelitis of Horses.*

Joest (1911) (Fig. 36) has described intranuclear inclusions in the ganglion cells of the brain of the horse suffering from meningo-encephalo-myelitis—Borna disease. The inclusions, which are found more especially in the cells of Ammon's horn, are very similar to those described in experimental herpetic meningo-encephalitis in the rabbit. They are strongly eosinophilic. Other observers have failed to find these inclusions.

(12) *Virus III Infection of Rabbits.*

In the course of an attempt to produce varicella lesions in the testicles of the rabbit by inoculation with human varicella material, Rivers and Tillett (1923–4) (Fig. 37) isolated a virus from the rabbit's testicle which on inoculation into the skin or cornea of the rabbit gave rise to lesions characterized by the presence of intranuclear inclusion bodies. Subsequently, Miller, Andrewes and Swift (1924) inoculated rabbits' testicles with blood and joint fluids from rheumatic fever patients. They obtained the same virus which later Andrewes and Miller (1924) (Fig. 38) were able to find in the testicles of apparently normal rabbits.

(13) *Paravaccinia.*

The condition now known as paravaccinia was originally described by Dauvé and Larue (1892) as "vaccine rouge." They noted that occasionally after the use of calf-lymph for inoculation an anomalous reaction took place. The clinical symptoms have been described by von Pirquet (1915–16), and the

cytological changes by Lipschütz (1919–20) (Fig. 39), who reported both cytoplasmic changes and intranuclear inclusions similar to those found in herpes.

(14) *Intranuclear Inclusions Associated with Diseases of Unknown Ætiology.*

Jesionek and Kiolemenoglou (1904) (Fig. 40) described protozoan-like structures in the kidneys, lungs and liver of an eight-month syphilitic stillborn fœtus. In the kidneys the cells containing the inclusions were irregularly scattered through the connective tissue of the cortex often in groups of from ten to forty. In the liver and lungs they occurred singly; in the lungs they were found free in the bronchi and alveoli. Shortly after the appearance of this paper, Ribbert (1904) (Fig. 41) recalled that he had seen similar bodies twenty years before in the kidneys of a syphilitic newborn fœtus, and in the parotid of a year-old non-syphilitic child. Loewenstein (1907) studied the parotids of thirty children from two months to two years old, and found inclusions in four. Pisanò (1910) described inclusions in the nuclei of the cells of the kidneys, liver, and more rarely of the lungs in a stillborn syphilitic fœtus. Mouchet (1911) found similar bodies in the bile-ducts of an eight-day syphilitic infant with icterus. Smith and Weidman (1910) regarded as an amœba the intranuclear bodies which they found in the liver and kidney of a newborn infant. The supposed parasites were surrounded by an inflammatory tissue containing lymphoid and polymorphonuclear leucocytes. The same authors (1914) described a second similar case, a two-months-old child dying of pneumonia, its Wassermann reaction being negative. Goodpasture and Talbot (1921) (Fig. 43) described the case of a child with acidophilic intranuclear bodies in the alveoli of the lungs, in the chronically inflamed bronchi, and in the glomeruli of the kidneys. De Lange (1922) recorded similar findings in the kidney of a three-day-old icteric infant, while Müller (1922) reported three cases—(i) a stillbirth, (ii) an eight-weeks-old child with hydrocephalus and interstitial nephritis, and (iii) a two-months-old child with congenital syphilis. The inclusions were only present in the kidneys. Von Glahn and Pappenheimer (1925) (Fig. 42) recorded intranuclear inclusions in the organs of a man aged 36 years dying with an abscess of the liver and ulcerative colitis, pleurisy and lobular pneumonia. The intranuclear bodies were found in the cells of the intestine, liver and lung. The bodies showed little evidence of internal structure, and by no staining method was it possible to identify minute granules within the inclusions. The staining reactions were similar to those of herpetic inclusions, but in addition the following reactions were obtained:

Stain.	Inclusion.
Mallory's acid fuchsin (aniline blue and orange G)	Bright red.
Gram's stain (gentian violet) . . .	Unstained.
Levaditi's stain	Chestnut brown.
Bensley's modification of Altmann's mitochondrial stain	Red.

(15) *Cellular Inclusion Bodies in the Guinea-pig.*

Jackson (1920), while examining guinea-pigs, found that in 26 out of 48 animals there were large cells resembling amœbæ in the salivary glands. The ducts of the serous portions of the glands were the favourite site of the "parasite," but they were occasionally found in the ducts of the mucous glands, surrounded in most cases by round-celled infiltration. Goodpasture and Talbot (1921) (Fig. 43) also examined the salivary glands of guinea-pigs, and recorded similar bodies which they regarded as identical with the protozoan-like cells recorded previously in infants.

Recently Kuttner and Cole (1926) have examined the submaxillary glands of 55 adult guinea-pigs, and have found acidophilic intranuclear inclusion bodies in 82 per cent. Inoculation of the salivary glands of old guinea-pigs into the brains, testicles and submaxillary glands of young guinea-pigs has given rise to a fatal meningitis. Inclusion bodies were found in the brain and testicles of the infected animals.

(16) *Kurloff Bodies.*

It should be noted that certain of the large mononuclear cells in the blood of guinea-pigs contain acidophilic inclusions in the cytoplasm. These inclusions, known as "Kurloff bodies," have by some writers been regarded as Chlamydozoa. They are also fairly numerous in the large mononuclears of the sheep, while analogous inclusions have been noted in the mononuclears of rats and fowls.

(17) *Foot and Mouth Disease.*

Gins (1922) (Fig. 44) described small intranuclear corpuscles in the lesions of foot and mouth disease on the tongue of the guinea-pig. The inclusions, which stain deep red with Giemsa, are never larger than 1·5 μ, and are situated near the nucleolus. They are said to be most numerous two days after infection. In addition this author has described cytoplasmic masses similar to those in the corneal cells of the rabbit inoculated with vaccinia or variola.*

(18) *Warts.*

Lipschütz (1924) has described intranuclear inclusions in the cells of infective warts in man, while Ullmann (1924) (Fig. 45) has figured closely similar bodies in the cells of the infective warts of dogs. The inclusions are basophilic, and are in all probability a stage in the degeneration of the nucleoli. They can scarcely be regarded as of a specific character.

(19) *Polyhedral and other Ultravirus Diseases of Caterpillars.*

Paillot (1926) (Figs. 47, 48 and 49) classifies the following diseases of insects as due to ultramicroscopic viruses:

(*a*) Polyhedral diseases associated with the presence of polyhedral crystals within the cell nuclei.

* F. Ruhle (*Archiv f. Tierheilkunde*, 1926, **54**, 197) records the finding of inclusion bodies similar to those described by Gins in the epithelial cells of the tongue of the normal guinea-pig and cow.

(i) "La grasserie" of the caterpillar of the Silkworm Moth—*Bombyx mori*. The fat cells are attacked.
(ii) Polyhedral disease of the caterpillar of the Black Arches Moth—*Hymantria monacha*.
(iii) "Wilt disease" of the caterpillar of the Gypsy Moth—*Hymantria dispar*.

(*b*) "Grasserie" of the caterpillar of the large cabbage white butterfly—*Pieris brassicæ*—unassociated with any cellular inclusions in the fat-cells.
(*c*) A nuclear disease of the caterpillar of *Pieris brassicæ* associated with the presence of refringent rings in the cytoplasm of blood and fat-cells: the refringent rings are said to be derived from the mitochondria.

The following other diseases may also be mentioned as having been associated with cytoplasmic inclusions. Unfortunately the original papers are either unaccompanied by illustrations or those provided convey nothing.

(20) *Fowl Plague.*

Kleine (1905) and Schiffmann (1906) have recorded the finding of inclusions in the ganglion cells of the brain not unlike Negri bodies.

(21) *Swine Fever.*

Uhlenhuth and Böing (1910) have described Chlamydozoa in the swollen epithelial cells of the conjunctiva.

(22) *African Horse Sickness.*

Kuhn (1911) has described inclusions in the renal epithelium.

(23) *A Disease of Fowls.*

Macfie (1914) (Fig. 50) and Adler (1925) have discovered in Nigeria and Palestine a disease of fowls with symptoms not unlike those of fowl spirochætosis. No bacteria or other organisms could be isolated from the blood, but in all varieties of leucocytes, except eosinophils and mast-cells, there were granular bodies, which when stained by Giemsa's method assumed the same tint as the nuclei of malarial parasites. The blood was infective to healthy fowls. The inclusions are regarded as Chlamydozoa. Filtration experiments were not carried out.

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Introduction.

* Denotes that the paper has been abstracted pictographically.

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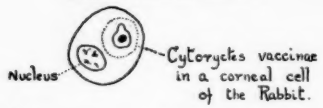
FIGURES.

NOTE.—All figures have been drawn to scale, with the exception of Figs. 20, 21 and 22, which have been reduced by one-third. In reproduction the degree of reduction is indicated within square brackets [ ] after the number of the figure.



Cytoryctes vaccinae: fixed alcohol-sublimate: stained. carmine.

Cytoryctes vaccinae in a corneal cell of the Rabbit.

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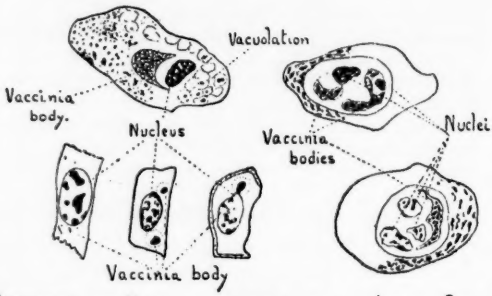
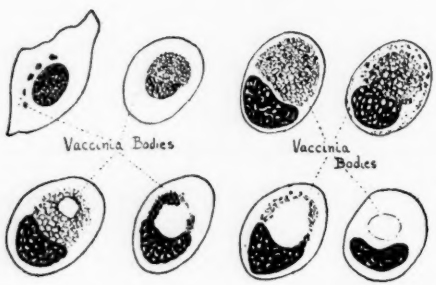
FIG. 1. [⅔]

— Vaccinia bodies in epidermal cells of Calf.



COPEMAN AND MANN. 28th REPORT LOCAL GOVT. BOARD 1898-9 505.

FIG 2 [⅔]



The Evolution of Vaccinia bodies in the Rats Cornea. Klatsch method Nocht-Romanowsky stain.

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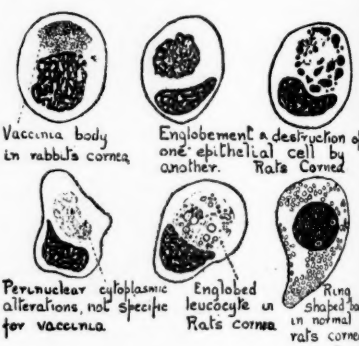
FIG 3. [⅔]


Vaccinia body in rabbit's cornea
Englobement a destruction of one epithelial cell by another. Rats Cornea
Perinuclear cytoplasmic alterations, not specific for vaccinia
Englobed leucocyte in Rats cornea
Ring shaped bodies in normal rats cornea

Development of Vaccine bodies in the Corneal cells of the Rabbit fixed Zenker stained Azur I

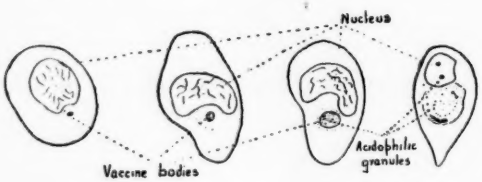


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FIG. 5 [½]

Conditions simulating Vaccinia bodies in Corneal Cells Klatsch method

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FIG. 4 [½]

—Vaccinia Bodies in corneal cells.—

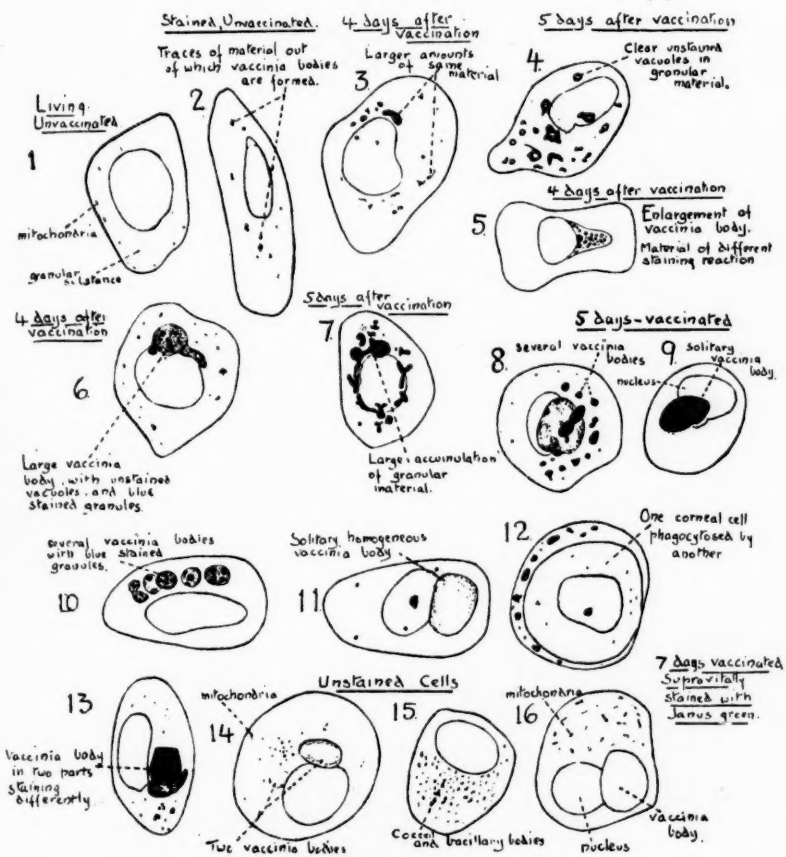
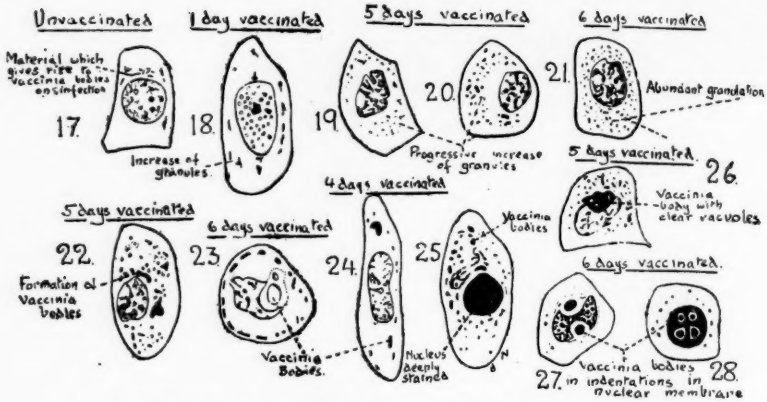


FIG. 6. [3/3]



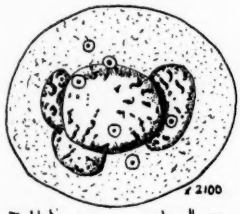
Histological Technique Employed

No. of Fig.	Fixative	Staining Method	No. of Fig.	Fixative	Staining Method
1	none	none	15	none	none
2	"	Stained intra vitam with brilliant cresyl blue, 2B.	16	"	Janus green B.
3	"		17	Zenker.	Giemsa
4	"		18	Zenker without acetic acid	Giemsa
5	"		19		
6	"		20		
7	"		21	"	"
8	"		22	Zenker	Giemsa.
9	"		23	Zenker	Wright
10.	"		24	Zenker	cartol-fuchsin and acid violet
11.	"		25	Acetic sublimate.	Giemsa
12.	"		26	Zenker.	Giemsa.
13	"		27	Zenker	Giemsa
14	"	none	28	Giemsa sublimate	Giemsa.

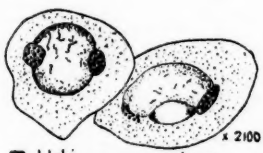
COWDRY, JOUR. EXPT. MED., 1922, VOL. XXXVI. 666.

FIG. 7. [2/3]

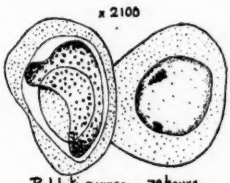
Stages in the development of Guarnieri bodies in the cornea & skin of the Rabbit: fixed Champy: stained eosin-haematoxylin.



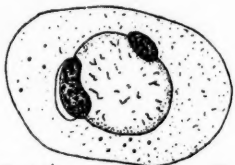
Rabbit's cornea: giant cell 24 hours after vaccination



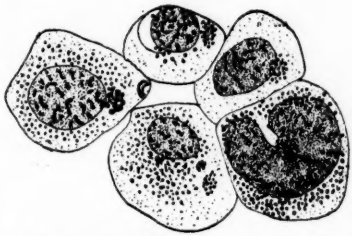
Rabbit's cornea: 70 hours after vaccination: Guarnieri bodies at the cell poles



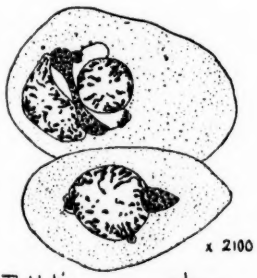
Rabbit's cornea: 70 hours after vaccination.



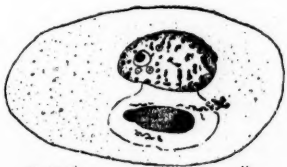
Rabbit's cornea 70 hours after vaccination x 2100



Disintegration of Guarnieri bodies in Rabbit's skin 100 hours after vaccination x 2100



Rabbit's cornea 70 hours after vaccination

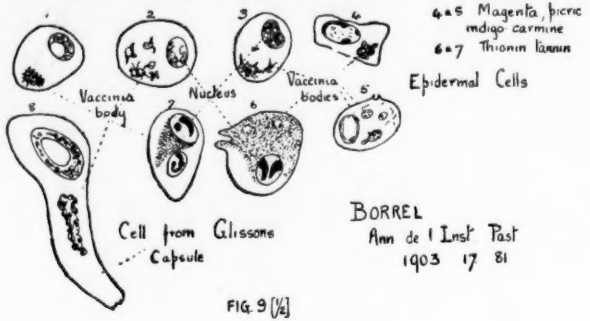


Rabbit's cornea 100 hours after vaccination x 2100

SCHÜTZ Zeit f Hyg u Infekt 1925-6 105 1

FIG. 8. [2/3]

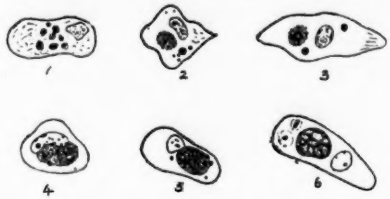
Cells of Sheep infected with Sheep pox stained 1-3 Laverans method
4-5 Magenta, picric indigo carmine
6-7 Thionin tannin



BORREL Ann de l'Inst Past 1903 17 81

FIG. 9 [½]

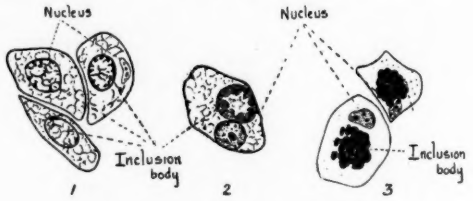
Cytoplasmic inclusions in epidermal cells of Hen with Fowl pox:
fixed Flemming's solution: stained- 1-3 Safranin, Indigo-carmine
Picric Acid: 4-6 Gentian violet, Vesuvin



APOLANT Virchow's Archiv f. path Anat. 1903 174. 86.

FIG. 10. [½]

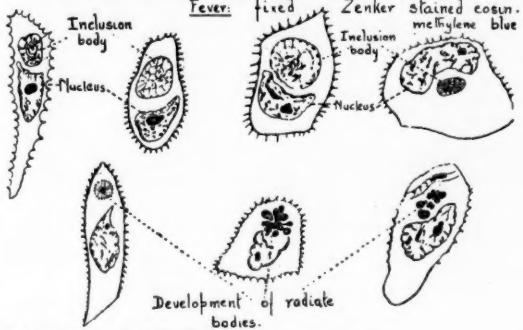
Cytoplasmic inclusions in epidermal cells of Pigeon with Fowl pox
fixed formalin: stained- 1 Iron haematoxylin and Orange G. 2 Alum haematoxylin and Orange G 3 Iron haematoxylin van Gieson



MICHAELIS. Zeitschrift f. Krebsforschung. 1903. 1 105.

FIG. 11. [½]

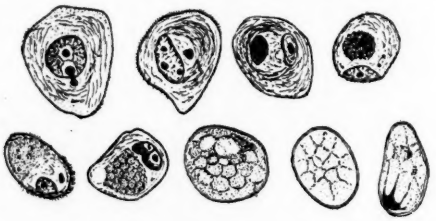
Inclusion bodies from epidermal cells in Scarlet Fever: fixed Zenker stained eosin. methylene blue



MALLORY. J. Med. Res. 1903-4. 10. 483.

FIG. 12. [½]

Molluscum contagiosum in the epidermal cells of Man fixed in alcohol or corrosive sublimate. stained Delafield's haematoxylin and eosin

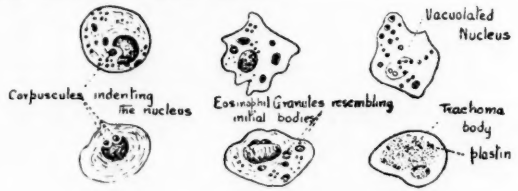


A. B MACALLUM J. Cutaneous a Genito Urin Dis 1892 10 93
FIG. 13. [½]

Stages in the development of Inclusion bodies in Trachoma in the cells of the Conjunctival Epithelium: stained Giemsa.

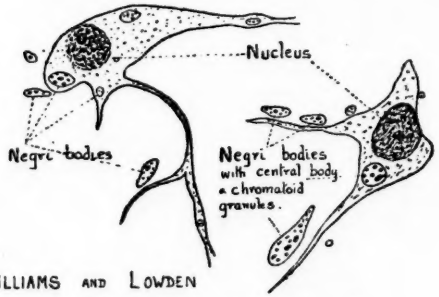
NOGUCHI and COHEN J Exp. Med. 1913 18 572.
FIG. 14. [½]

Epithelial cells of the conjunctiva from Trachoma in Man. fixed Schaudinn stained Giemsa



SOLOVIEF Archives des Inst Past de l'Afrique du Nord 1921. 1 388.
FIG. 15 [½]

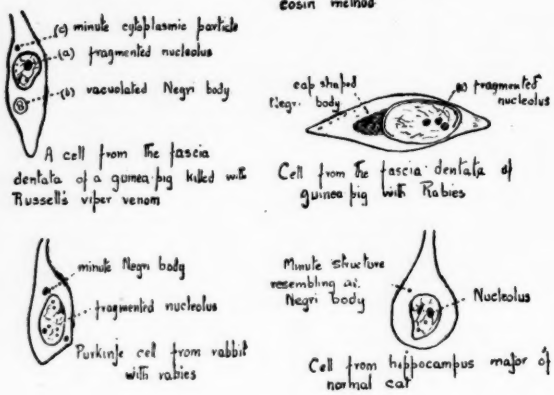
Negri bodies in cells of Ammon's horn of Dog with Rabies



WILLIAMS AND LOWDEN
J. Infect. Dis 1906 3 452
FIG. 16. [½]

Negri bodies in the brain cells in Rabies compared with inclusions unassociated with this disease:-

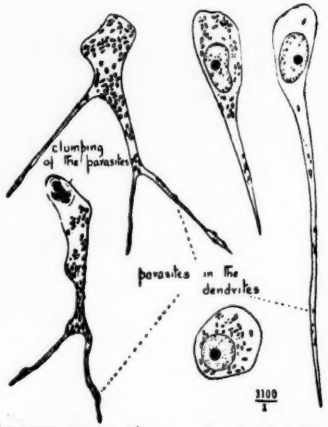
stained Mann's methyl-blue eosin method


(c) minute cytoplasmic particle
(a) fragmented nucleolus
(b) vacuolated Negri body
A cell from the fascia dentata of a guinea pig killed with Russell's viper venom
cap shaped Negri body
(a) fragmented nucleolus
Cell from the fascia dentata of guinea pig with Rabies
minute Negri body
fragmented nucleolus
Purkinje cell from rabbit with rabies
Minute structure resembling a Negri body
Nucleolus
Cell from hippocampus major of normal cat
yellow vesiculated mass
nucleolus with yellow staining centre
Human cortical cell from a case of hydrophobia

ACTON and HARVEY. Parasitol. 1911 4 255

FIG. 17. [½]

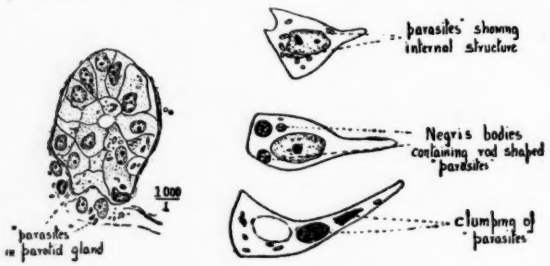
Nerve cells from the cerebral cortex and Ammons horn of a dog with Rabies containing "Encephalitozoon rabiei" fixed by the alcohol-acetic-sublimate method of Gilson stained Manns method



MANOUELIAN and VIALA Ann de l'Inst Past 1924 38 258

FIG. 18. [½]

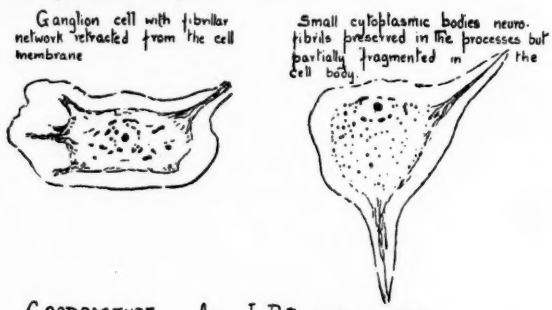
"Encephalitozoon rabiei" in the nerve cells of Ammons horn & in the salivary gland of dog with Rabies stained Manns method



MANOUELIAN and VIALA Ann de l'Inst Past 1924 38 258

FIG. 19. [½]

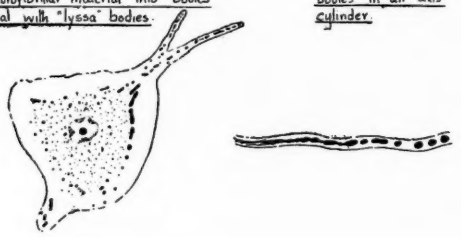
Stages in the development of Negri bodies in the brain of the Rabbit stained by carbol anilin fuchsin


Ganglion cell with fibrillar network retracted from the cell membrane
Small cytoplasmic bodies neuro-fibrils preserved in the processes but partially fragmented in the cell body.

GOODPASTURE. Amer J Path 1925. 1. 547
FIG. 20. [½]

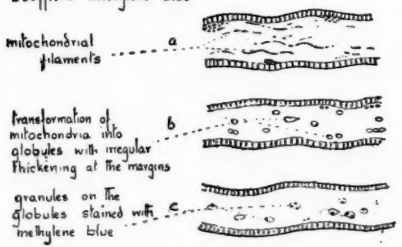
Ganglion cell showing final break up of the neurofibrillar material into bodies identical with "lyssa" bodies.

Formation of hyaline bodies in an axis cylinder.



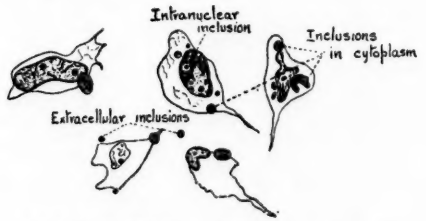
GOODPASTURE Amer. J Path 1925 1 547
FIG. 21. [½]

Stages in the development of Negri bodies in the brain of the Rabbit fixed Zenker stained a. & b. fuchsin, methyl green c. acid fuchsin & Loefflers methylene blue


mitochondrial filaments
a
transformation of mitochondria into globules with irregular thickening at the margins
b
granules on the globules stained with methylene blue
c

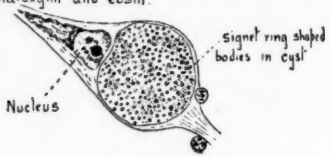
GOODPASTURE Amer J Path. 1925. 1 547
FIG. 22. [½]

Inclusion bodies in ganglion cells of Ammon's horn of dog with Distemper stained eosin-methylene blue



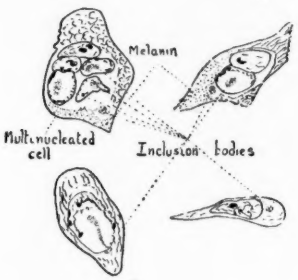
LENTZ. Zeitschr. f. Hygiene u. Infect. 1909 62. 63.
FIG. 23. [½]

Ganglion cell from brain of dog with distemper containing a cyst stained Heidenhain's haematoxylin and eosin.



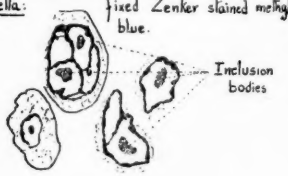
KANTOROWICZ and LEWY Archiv f Wiss u prak Tierheilkun 1922-3. 49 137 FIG. 24. [½]

Epidermal cells from Varicella in Man: fixed in Zenker stained eosin methylene blue



TYZZER. J. Med Res 1905-6 14 361. FIG. 25. [½]

Nuclear inclusions in epidermal cells of man: Varicella: fixed Zenker stained methylene blue.



Varicella inclusion bodies in the epidermal cells of the Rabbit.

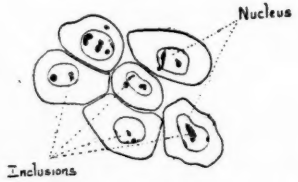
fixed Alcohol sublimate : stained 1, 3 and 4 Giemsa 2 Manson's stain
1 Inclusion in almost normal epidermal cell
2 Disintegrating inclusion body.
3 Giant cell with inclusion body.
4 Inclusion body with internal granules.

GINS Zeitschrift f Hygiene 1918 86. 299 FIG. 26. [½]

Nuclear inclusions in testicle of Vervet injected with human Varicella material

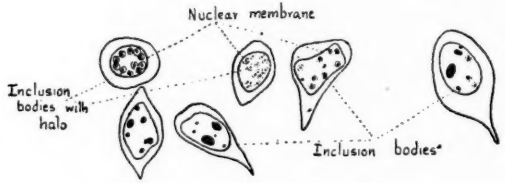
RIVERS J Exp. Med 1926 43 275. FIG. 27. [½]

Herpetic Inclusions
stained Giemsa



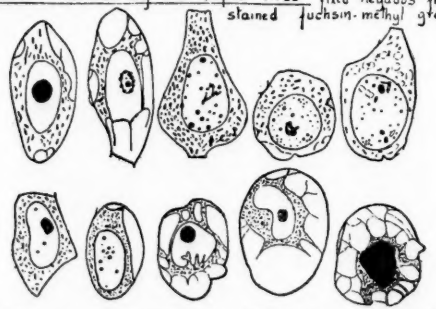
LIPSCHUTZ Archiv. f. Dermat. u. Syph. 1921. 136. 428.
FIG. 28. [½]

Inclusion bodies from Nerve Cells of Rabbit's brain infected with Encephalitis lethargica stained Mann's method.



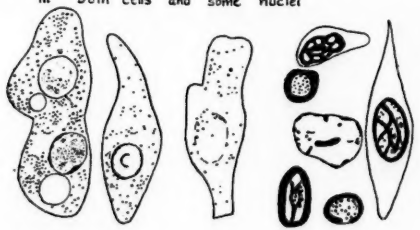
LEVADITI "Poliomyélite et Encephalite" 1922 Paris.
Ectodermoses neurotropes. a Ann de l'Inst. Past. 1922 36 77.
FIG. 29. [½]

Nuclear inclusions in nerve cells of nucleus caudatus of Rabbit injected intracerebrally with Herpes virus: fixed Regauds fluid: stained fuchsin-methyl green.



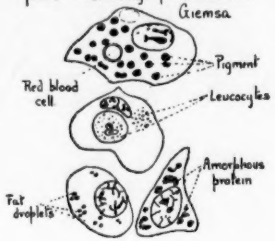
COWDRY AND F.M. NICHOLSON J. Exp. Med 1923 38. 695.
FIG. 30. [½]

Cells and isolated nuclei from brain of Rabbit injected intracerebrally with Herpetic virus: air dried smears stained Giemsa: dark blue granules in both cells and some nuclei

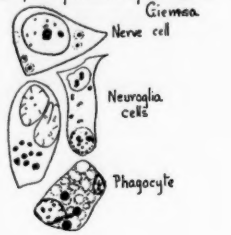


COWDRY AND F.M. NICHOLSON. J. Exp. Med. 1923. 38. 695.
FIG. 31. [½]

Macrophages from meninges of Rabbit with Herpetic meningo-encephalitis. fixed Flemmings fluid stained Giemsa

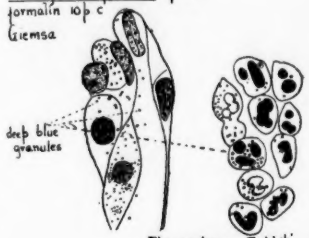


Inclusions in brain cells of Rabbit infected intracerebrally with normal brain. fixed formalin 10 p c stained Giemsa.

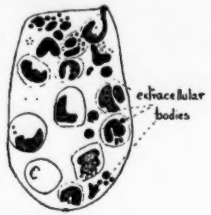


COWDRY AND F M NICHOLSON. J Exp. Med 1923 38 695.
FIG. 32. [½]

Capillary and neuroglia from brain of Rabbit Herpetic meningo-encephalitis fixed formalin 10 p c Giemsa



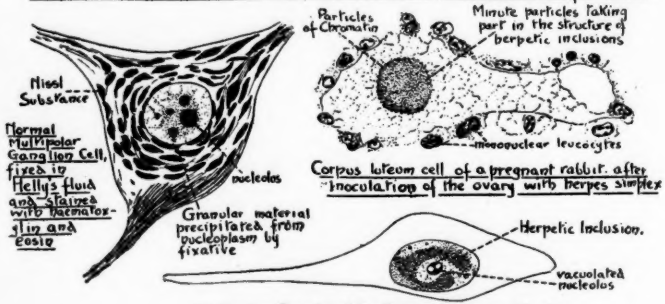
Leucocytes and extracellular bodies in brain of Rabbit Herpetic meningo-encephalitis



Phagocytes in Rabbit's cornea: Zenker. stained Giemsa

COWDRY AND F M NICHOLSON J Exp Med 1923 38. 695
FIG. 33. [½]

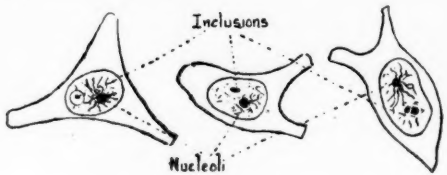
Intranuclear Inclusions in Experimental Herpetic Lesions of Rabbit's.


Particles of Chromatin
Minute particles taking part in the structure of herpetic inclusions
Nissl Substance
Normal Multipolar Ganglion Cell, fixed in Helly's fluid and stained with haematoxylin and eosin
nucleolus
mononuclear leucocytes
Corpus luteum cell of a pregnant rabbit. after inoculation of the ovary with herpes simplex
Granular material precipitated from nucleoplasm by fixative
Herpetic Inclusion.
vacuolated nucleolus

Multipolar Ganglion Cell after inoculation with the virus of Herpes simplex

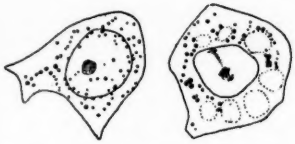
GOODPASTURE, AM. JOUR. PATH. 1925, 1, 1-9
FIG. 35. [½]

Nucleolar Inclusions in the ganglion cells of Ammons horn from the brain of a horse with Meningo encephalo myelitis stained by Stutzers method



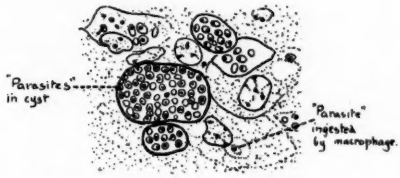
JOEST. Zeitschr f Infekt Krankheit der Haust. 1911 10 293.
FIG. 36 [½]

Herpetic Meningo-Encephalitis in Rabbits



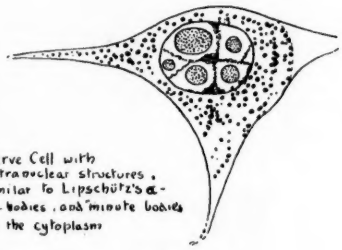
"Minute bodies" in nerve cells (×1600)

"Protozoan-like" Parasites in Spontaneous Encephalitis of Rabbits



From Brain Section (×950)

Da Fano. Jour Path and Bact 1924, XXVII. 339-336

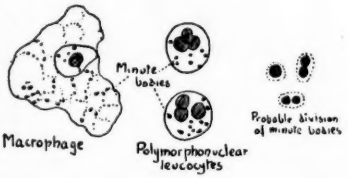


Nerve Cell with intranuclear structures, similar to Lipschütz's α-β-bodies, and minute bodies in the cytoplasm

"Minute bodies" in polymorphonuclear leucocytes in acute Encephalitis

(× 2500)

Da Fano Jour Path and Bact 1924. No 11-26



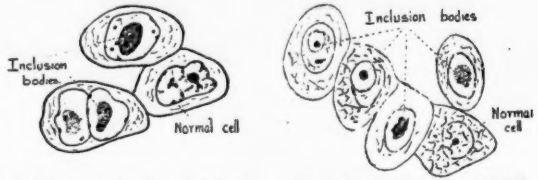
Da Fano, Jour Path and Bact, 1923, Vol XXVI, 85-115

Minute bodies in Chronic Epidemic Encephalitis in a young child

Da Fano and Ingleby Jour. Path. and Bact 1924, XXVII. 349-365.

FIG. 34.[⅔]

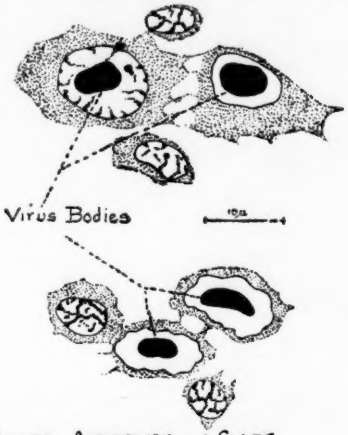
Nuclear inclusions in cells of Rabbit inoculated with Virus III. fixed Zenker stained - Eosin-methylene blue Giemsa



Endothelial leucocytes from testicle of Rabbit inoculated with Virus III. Corneal cells from eye of Rabbit inoculated with VIRUS III

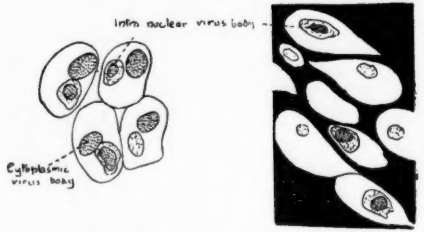
RIVERS AND TILLETT J. Exp. Med. 1924 40. 281
FIG. 37. [illegible]

Nuclear inclusions in cells of Rabbits testicle fixed Zenker stained eosin methylene blue. (drawn from a section lent by the authors)



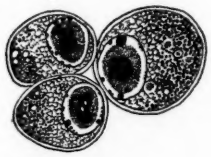
MILLER, ANDREWES and SWIFT
J Exp. Med. 1924 40. 773 & 789
FIG. 38. [illegible]

Cytoplasmic and Intranuclear Inclusions in Paravaccinia



LIPSCHUTZ, ARCH. DERMAT. und SIPHILIS, 1919-20, 126
FIG. 39. [illegible]

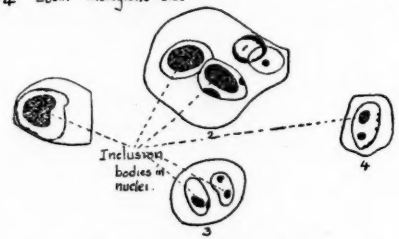
Nuclear inclusions in the renal epithelium of a syphilitic fœtus stained Hæmatoxylin and eosin



JESIONEK and KIOLEMENOGLOU Münch. med Woch 1904 51 1905

FIG. 40. [½]

Intranuclear inclusions in the cells of the lung of an adult dying with obscure symptoms stained 1 Hæmatoxylin and eosin 2-4 Eosin methylene blue



VONGLAHN AND PAPPENHEIMER Amer. J Path 1925. 1. 445

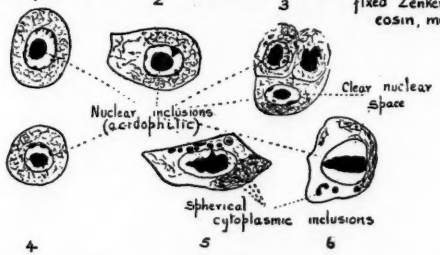
FIG. 42. [½]

Intranuclear inclusions in (a) the kidneys (b) the salivary glands of infants

RIBBERT Centralbl. f allgemeine path u path Anatomie 1904 15. 945

FIG. 41. [½]

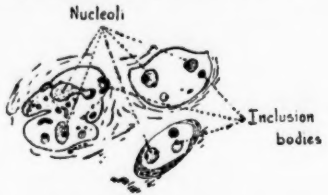
Cytoplasmic inclusions unassociated with any known virus infection
1-4 Cells from bronchial exudate of infant dying with broncho-pneumonia
5 & 6 Cells in wall of serous salivary gland of Guinea pig fixed Zenker stained eosin, methylene blue



GOODPASTURE AND TALBOT Amer. J. Diseases of Children 1921 21. 415

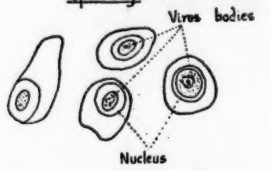
FIG. 43. [½]

Inclusions in the epidermal cells of the tongue of the Guinea pig with Foot & Mouth disease: stained Giemsa



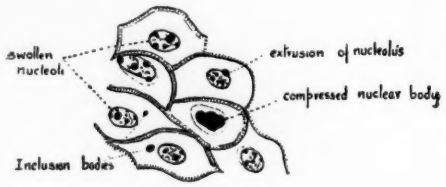
GINS. Centralbl f Bakt. 1922 88 265
FIG. 44. [½]

Cells from Infective Warts of Dog: stained Giemsa.



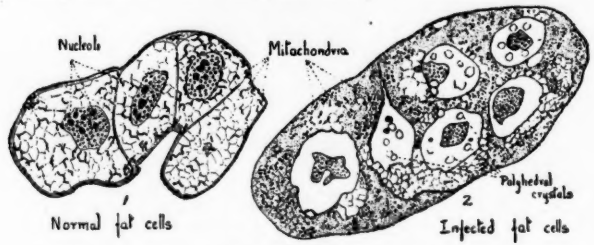
ULLMANN. Acta Medica Laryn. 1923-4 5 317
FIG. 45. [½]

Epidermal cells from Verruca vulgaris - infective warts in Man.



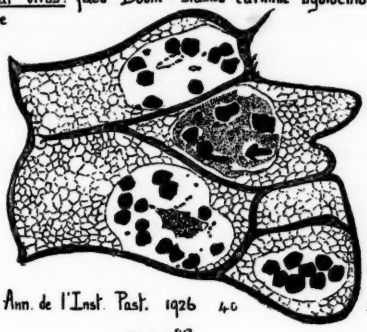
KYRLE "Histo biologie der Haut." Berlin 1925
FIG. 46. [½]

Fat cells of the Caterpillar of the Silk Worm Moth (Bombyx mori) infected with the polyhedral virus — "grasserie" fixed (1) in Regaud's sol (2) in formol saline stained acid fuchsin, aniline and toluidin blue (Kull's method)



PAILLOT Ann de l'Inst Past 1926 40 314
FIG. 47. [½]

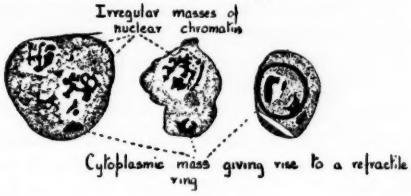
Fat cells of the Caterpillar of the Silk Worm Moth (Bombyx mori) infected with the polyhedral virus: fixed Bouin stained carmine hydrochloride picric indigo-carmine



PAILLOT Ann. de l'Inst. Past. 1926 40 314

FIG. 48. [½]

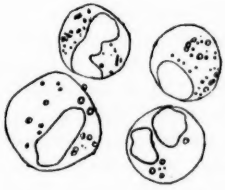
Micronucleocytes from the blood of the caterpillar of the large Cabbage White Butterfly (Pieris brassicae) inoculated with blood from a caterpillar infected with nuclear disease - "maladie du noyau" stained Giemsa.



PAILLOT Ann de l'Inst Past 1926 40. 314

FIG. 49. [½]

Inclusions in the leucocytes of fowls suffering from an acute infective disease stained Giemsa



MACFIE Annals of Trop Med and Parasitol 1914 8 439

FIG. 50. [½]

THE ULTRA-MICROSCOPIC VIRUSES: II.—THE CYTOLOGY OF FOWL-POX.

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From the Laboratories of the Imperial Cancer Research Fund, London.

Received for publication August 9th, 1926.

THE present paper represents part of an investigation which is now being undertaken in these laboratories into the disease known as "Fowl-pox." The condition has also been described as epithelioma contagiosum and molluscum contagiosum of birds. Both these terms must be regarded as misnomers, for the disease has nothing in common either with the epitheliomata or with molluscum contagiosum in man. On the other hand there is considerable evidence to show that the virus of fowl-pox, if not identical, is at least related to the virus of vaccinia.

From the cytological point of view interest has largely centred in the curious cell inclusions found in the infected epidermal cells in this disease. They were first described as gregarine parasites, and since then have been the subject of many investigations. Up to the present, however, no attempt has been made to study the origin and nature of these inclusions by modern cytological methods.

The strain of virus used in the present investigation was obtained from a spontaneous epidemic of fowl-pox which occurred on a large poultry farm near St. Albans, Hertfordshire. The virus has been maintained for more than a year under laboratory conditions on the scarified skin of chickens from one to two weeks old, a fresh passage having been made every ten to fourteen days.

TECHNIQUE.

The characteristic lesions in the skin were studied in fixed and stained preparations, and also "*intra vitam*" from smears. The histological methods principally employed were the modified Kopsch method for the Golgi apparatus and the Schridde method for mitochondria. Preparations made by the former method were examined unstained, or after staining with neutral red. Schridde sections were stained by the iron-hæmatoxylin acid-fuchsin method described elsewhere (Ludford, 1926). Blood-films were prepared and stained by the Giemsa method; smears were also fixed in osmic vapour and stained in various ways, including the iron-hæmatoxylin method. All attempts to identify a micro-organism in blood-smears were unsuccessful. Similarly it was not possible to detect any cytological peculiarities in the cells of the liver of infected birds, despite the fact that both the blood and liver are highly infective when applied to the scarified skin of healthy chickens. We are therefore forced to the conclusion that the virus when present in the blood is of ultra-microscopic proportions.

THE EARLY STAGES OF INFECTION OF EPIDERMAL CELLS.

Fig. 1 shows a normal cell from the lowest layer of the stratum mucosum of the epidermis. At its distal end are unstained areas representing the position occupied by the Golgi apparatus, which is not impregnated in this Schridde preparation. At the proximal end of the cell, *i. e.* bordering on the dermis, are the mitochondria (*m*). The first indication of infection is the presence of minute vacuoles, such as the two which are shown in Fig. 2. They arise in the cytoplasm at the proximal end of the cell. Fig. 3 shows an almost identical stage in a Kopsch preparation. Here the Golgi apparatus (*GA*) is in its normal position at the upper pole of the nucleus. No mitochondria are shown, but two droplets each with very minute granules at its periphery are recognizable. These fine granules are visible owing to their being impregnated by the osmic acid. Fig. 4 shows a cell with a much larger vacuole or colourless sphere, provided with the same osmophil particles. At later stages of infection these droplets or vacuoles reduce the osmic acid, and appear of a brownish colour in unbleached sections, instead of colourless as at first. When a few vacuoles only are present in the cell, mitosis is quite common. Thus vacuoles are present in the cell in the telophase shown in Fig. 5, where the mitochondria are stained, while in Fig. 6 there are both virus inclusions and Golgi bodies. The droplets in the latter figure are, however, much larger, and have reduced the osmic acid of the fixative so as to appear brown in colour. Examination of sections of normal skin showed no evidence of any preformed substance in the epidermal cells from which the earliest vacuoles were derived.

DEVELOPMENT OF VIRUS BODIES DURING KERATINIZATION OF THE CELLS.

In the examination of sections, as one passes from the lowest layer of cells of the epidermis towards the surface, the cytological features presented are the expression of two processes:

(*a*) Keratinization—see diagram.

(*b*) The progressive infection of the cells by the virus.

During keratinization (Ludford, 1924) the cytological changes that occur are:

(i) The Golgi apparatus breaks up; the fragments are dispersed in the cytoplasm and finally become indistinguishable.

(ii) The mitochondria usually become vesicular and then disappear. Sometimes they form filaments; these, together with the so-called epidermal fibrillæ, may persist in the keratinized cell, or they may disintegrate and disappear.

(iii) There is an extrusion of nucleolar material into the cytoplasm to form granules of keratohyalin (stratum granulosum of the skin).

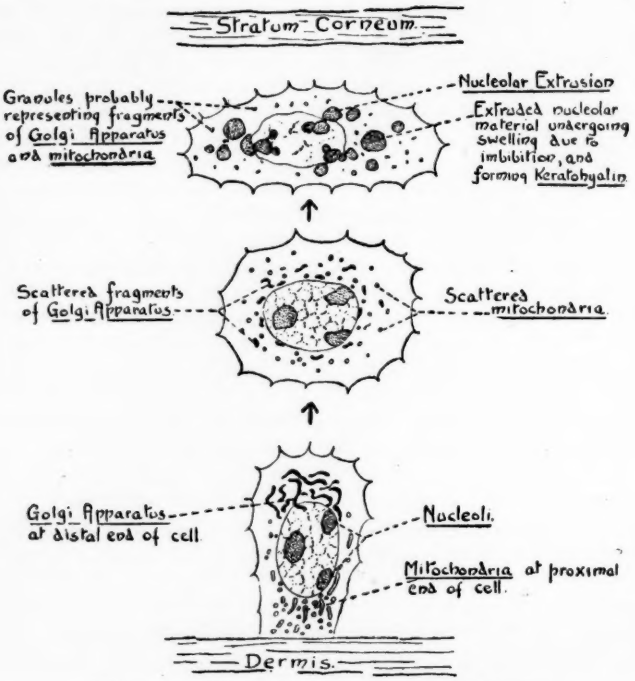
It is important to distinguish the changes due to keratinization from those set up by the virus. There is no doubt that confusion has arisen in the past owing to failure to differentiate between the two processes.

The changes brought about by the virus in the course of progressive infection are as follows:

(*a*) The droplets or vacuoles already referred to increase in number and enlarge (Figs. 7, 8).

(*b*) The vacuoles coalesce so as to form a single large body—the so-called "molluscum body" (Figs. 11, 12).

(*c*) Many cells become hypertrophied, and at an early stage of infection there is often a complete reversal in the polarity of the Golgi apparatus (Figs. 9, 10).


Stratum Corneum
Granules probably representing fragments of Golgi Apparatus and mitochondria
Nucleolar Extrusion
Extruded nucleolar material undergoing swelling due to imbibition, and forming Keratohyalin
Scattered fragments of Golgi Apparatus
Scattered mitochondria
Golgi Apparatus at distal end of cell
Nucleoli
Mitochondria at proximal end of cell
Dermis

Scheme of keratinization in normal epidermal cells.

Fig. 7 shows the increase in number of the droplets. The appearance here suggests that a budding, or fission, is taking place. Small granules attached to the surface of the vacuoles occur as in Figs. 3 and 4. In Fig. 7, however, these granules are stained with acid-fuchsin. Nucleolar extrusions are a notable feature of this cell.

Further enlargement of the vacuoles is shown in Fig. 8. The preparation from which this cell was drawn was bleached, otherwise the largest of the vacuoles would have been darkened by the osmic acid. The granules in the cytoplasm stained by acid-fuchsin represent mitochondria and fragments of the disintegrated epidermal fibrillæ.

Reversal in the polarity of the Golgi apparatus is shown in Figs. 9 and 10. The connective tissue of the dermis (*c*) is seen at the top of both of these figures. In the largest cell in Fig. 9 the Golgi apparatus has moved round to the

opposite pole of the nucleus to the normal and is intimately associated with the virus vacuoles, which here appear darkened by the osmic acid, the preparation from which it was drawn being unbleached. Other cells of this figure exhibit abnormalities in the polarity of the apparatus. The upper cell of Fig. 10 is considerably hypertrophied. Its Golgi apparatus shows reversal of polarity, and is at the same time very considerably enlarged. Vacuoles caused by the virus are visible. The lower cell of Fig. 10 has a greater number of vacuoles, and the Golgi apparatus is fragmented. Such an early disintegration is abnormal, and is probably caused by the action of the virus.

It has already been mentioned that the first indication of infection of a cell by the virus is the appearance in the cytoplasm of a minute vacuole. Even in unbleached osmic acid preparations such vacuoles appear colourless. The larger vacuoles are brownish in colour, while the largest, in unbleached sections, are deep black (Fig. 13). This coloration is very difficult to remove with turpentine; in most cases it is impossible to do so. It can, however, be removed by bleaching sections for 30 minutes to 1 hour in Cramer's alcohol-peroxide mixture. The appearance presented by Schridde preparations after bleaching in this way and staining with iron-hæmatoxylin and acid-fuchsin is very striking. Some of the vacuoles, usually only the smallest, remain colourless. In others what look like fine granules are visible inside the walls of the vacuoles. Fig. 11 illustrates this feature. It probably also represents the process of fusion of the vacuoles, which finally results in a single large body as is shown in Fig. 12. In this latter figure the granulation is clearly evident after bleaching and staining. The unbleached cell at the same stage of infection appears as in Fig. 13.

Just below the superficial keratinized zone of the lesions the virus inclusions present the appearance shown in Fig. 14, when unbleached. The normal structure of the cells is completely disorganized, partly as the result of keratinization, and partly owing to the large virus bodies present. The appearance of the cell shown on the left in Fig. 14 suggests that the single virus bodies so often seen in cells may arise from the fusion of smaller ones.

Within the keratinized zone of the lesion the presence of virus inclusions within incompletely keratinized cells results in a cyst-like appearance. This is represented in Fig. 15, which was drawn from an oblique tangential section that had been bleached before staining, so that the virus bodies look granular.

Secondary infection of the lesion by bacteria is of general occurrence. The bacteria occupy cavities in the keratin due to incomplete keratinization of the cells infected by the virus. Well-developed virus inclusions and bacteria rarely occur in the same cavity. Occasionally one sees badly stained granules amongst the bacteria: these are probably responsible for the destruction and replacement of the virus bodies (see Fig. 15).

THE STRUCTURE AND NATURE OF THE VIRUS INCLUSIONS.

As seen in Fig. 3, to the smallest of the vacuoles there are attached granules which are impregnated by the osmic acid technique; after bleaching they can be stained with acid-fuchsin.

It is impossible to be certain whether the larger vacuoles figured, *e. g.*, in

Figs. 7, 8 and 11 arise from an enlargement of the smaller ones or whether they are due to coalescence. The appearance seen in Fig. 7 suggests a process of budding, followed perhaps by coalescence. When a certain size has been attained, however, the vacuoles become surrounded by a layer of some material evidently lipoidal in nature. This reduces the osmic acid of the fixative and gives the black appearance in sections (Fig. 13). After removal of this impregnation by bleaching the inside of the virus bodies looks granular (Figs. 12 and 15). It is quite conceivable that this granular appearance is the optical expression of what is really an alveolar structure. The large virus body may conceivably be composed of numerous minute droplets. On altering the focus of the microscope the appearance of the spaces between these droplets would account for the granular appearance. There is, however, the other possibility that the virus body is actually granular in structure.

Whatever may be the correct interpretation of these pictures, there seems little doubt that the morphological sequence of the infective stages figured is indicative of the reaction of the cell to the virus rather than stages in the life-cycle of the actual organism.

DESCRIPTION OF FIGURES.

Abbreviations employed: *c*, Connective tissue of dermis; *ch*, chromosomes; *GA*, Golgi apparatus; *Gb*, Golgi bodies formed as the result of the breaking up of the reticulate Golgi apparatus; *m*, mitochondria; *nl*, nucleolus; *nx*, nucleolar extrusions; *k* denotes that the figure was drawn from a modified Kopsch preparation, either stained with neutral red or unstained; *s* denotes that the figure was drawn from a Schridde preparation stained by the iron-hæmatoxylin acid-fuschin method; *b* indicates that the preparation has been bleached with hydrogen peroxide.

FIG. 1.—Normal cell from the lowest layer of the stratum mucosum of the skin of the chick, showing mitochondria, *m* (*s*).

FIG. 2.—Similar cell showing the first indication of infection (*s*).

FIGS. 3 and 4.—Two epidermal cells at early stages of infection. The Golgi apparatus (*GA*) is in its normal position (*k*).

FIGS. 5 and 6.—Telophase of mitosis at early stages of infection. Fig. 5 shows mitochondria, *m* (*s*); Fig. 6 the Golgi bodies, *Gb* (*k*). Virus inclusions present in both cells.

FIGS. 7 and 8.—Epidermal cells showing the increase in size and in numbers of the vacuoles produced by the virus. Nucleolar extrusions (*nx*) are occurring in Fig. 7, and broken-up fibrillæ in addition to mitochondria are shown in Fig. 8 (*sb*).

FIG. 9.—Group of epidermal cells showing the reversal of the polarity of the Golgi apparatus, *GA*, and the formation of the virus inclusions in intimate relationship with the Golgi apparatus (*k*).

FIG. 10.—Two epidermal cells at early stages of infection. The upper cell is considerably hypertrophied and the Golgi apparatus is enlarged, and at the opposite side of the nucleus to what is normal. In the lower cell the Golgi apparatus is broken up into granules, *Gb* (*k*).

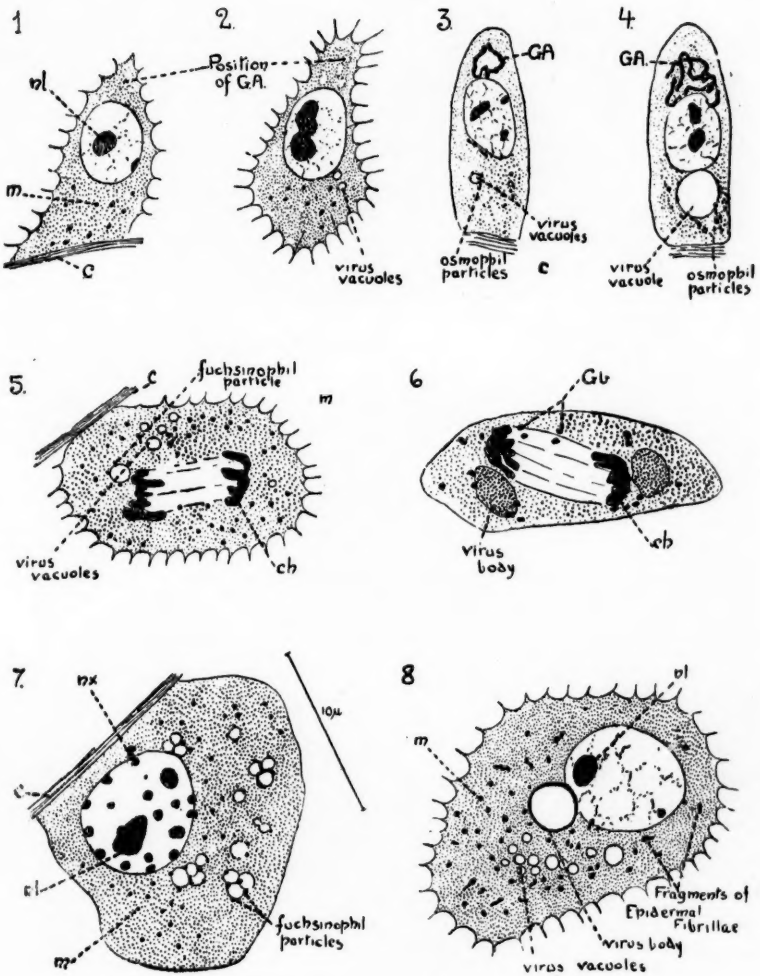
FIG. 11.—Cell with numerous virus inclusions, and the larger appearing granular around their peripheries (*sb*).

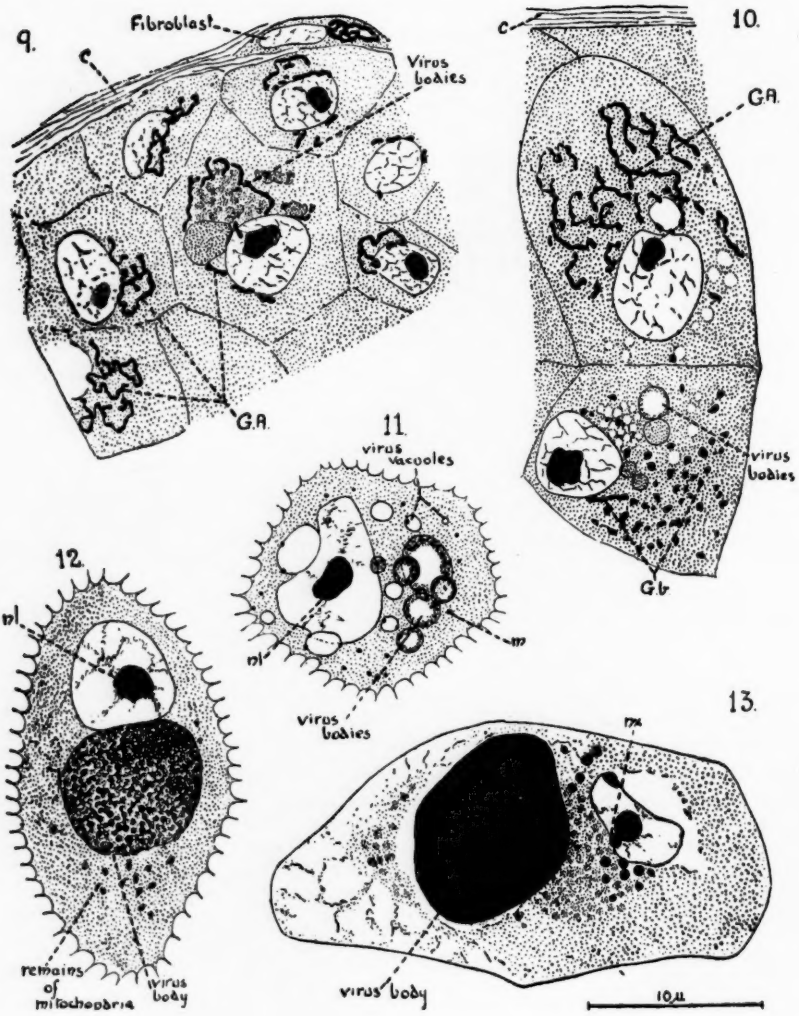
FIG. 12.—Cell with a single virus body presenting a finely granular structure (*sb*).

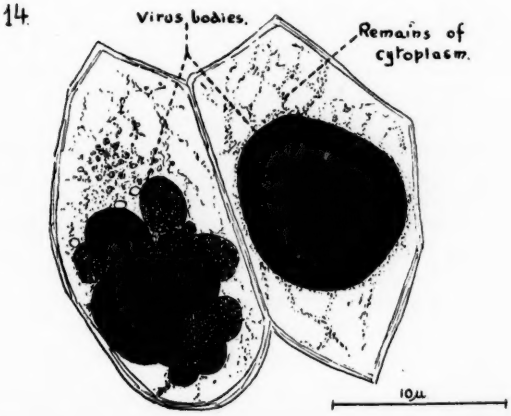
FIG. 13.—The virus body as it appears in unbleached sections. Nucleolar extrusions are figured in this cell (*s*).

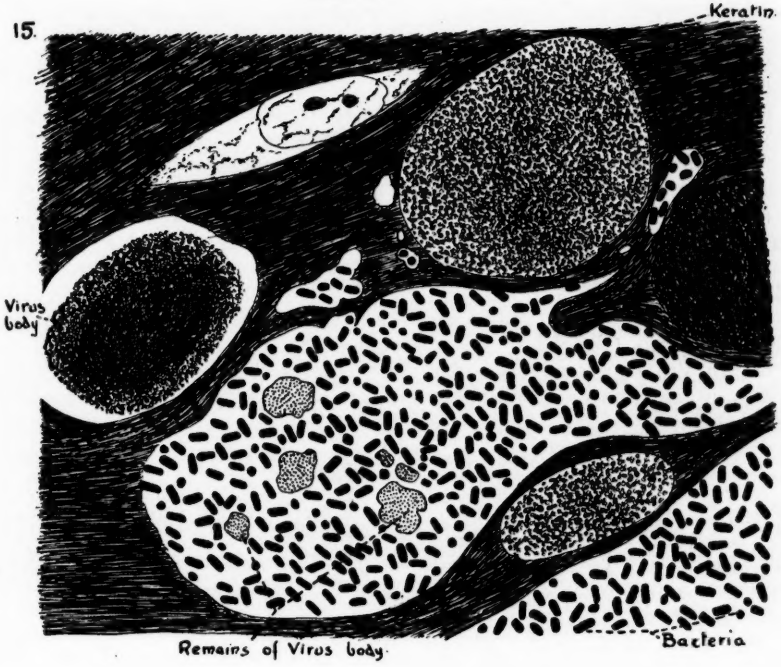
FIG. 14.—Two cells from the superficial keratinized zone of the lesion, showing how the virus bodies probably coalesce to form a single inclusion (*s*).

FIG. 15.—Oblique section tangential to the surface of the lesion, showing the cyst-like structure due to the presence of the virus bodies in the keratinized layer. Bacteria are seen occupying certain of the cavities similar to those filled by the virus inclusions (*sb*).


1
2.
3
4
vl
Position of G.A.
m
c
virus vacuoles
GA
virus vacuoles
osmophil particles
c
GA.
virus vacuole
osmophil particles
5.
f
fuchsinophil particle
m
virus vacuoles
ch
6
Gb
virus body
ch
7.
nx
10μ
c
vl
fuchsinophil particles
m
8
vl
m
Fragments of Epidermal Fibrillae
virus body
virus vacuoles


9.
Fibroblast
c
Virus bodies
G.A.
10.
c
G.A.
virus bodies
G.b
11.
virus vacuoles
m
m
virus bodies
12.
m
remains of mitochondria
virus body
13.
m
virus body
10 μ


14
Virus bodies.
Remains of cytoplasm.
10μ


15.
Keratin.
Virus body
Remains of Virus body.
Bacteria

DISTRIBUTION OF THE VIRUS BODIES.

We have already referred to the fact that the virus bodies have only been seen in the lesions in the epidermis—an occurrence which is probably related to the process of keratinization which is going on in these cells. The virus bodies become progressively larger as one passes from the lowest layer of cells of the epidermis towards the surface—that is, as the cells become more disorganized in the course of cornification. Furthermore, cells containing the large virus bodies (Fig. 14) are imperfectly keratinized—a condition which may be due to the products of cellular disruption contributing to the formation of the virus bodies, instead of becoming transformed into the sclero-proteid keratin. The disorganization of the cells incident upon keratinization possibly provides conditions for the virus to develop, as well as the materials essential for the formation of the characteristic virus bodies.

SUMMARY.

(1) The earliest indication of infection of an epidermal cell is the formation of a small vacuole, to the periphery of which small granules are attached (Figs. 2, 3 and 4).

(2) Mitosis is of common occurrence in cells containing such vacuoles (Figs. 5 and 6).

(3) These "virus" vacuoles increase in numbers and become enlarged. Some form of budding seems suggested (Figs. 7 and 8).

(4) Concurrently with enlargement the virus vacuoles become enclosed by a lipoidal lining and present a granular appearance internally (Fig. 11).

(5) Many cells become hypertrophied, and at an early stage of infection there is a complete reversal in the polarity of the Golgi apparatus (*GA*), which in many cases stands in intimate relationship to the virus bodies (Figs. 9 and 10).

(6) The virus bodies tend to coalesce (Figs. 11 and 14) and there results a single virus body (Fig. 12, bleached and stained; Fig. 13, unbleached).

(7) In the superficial cornified layer of the lesion virus bodies occupy cavities in the keratin. Similar cavities are secondarily infected with bacteria (Fig. 15).

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LUDFORD, R. J.—(1924) *Quart. J. Micr. Sci.*, **69**, 27.—(1926) *J. R. Micr. Soc.*, **46**, 107.

THE REACTION OF THE BLOOD IN EPILEPSY, WITH A DISCUSSION OF COLORIMETRIC METHODS OF ESTIMATING THE pH OF PLASMA.

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Received for publication July 13th, 1926.

THE low hydrogen ion concentration of the plasma found by Bigwood (1924) in active genuine epilepsy plays an important part in the theory he has put forward of the genesis of epileptic fits. We have discussed one aspect of this theory—that relating to calcium ionization—elsewhere (*Biochemical Journal*, in press); here we propose to consider the actual hydrogen ion concentration.

It has been suggested to us by Prof. Cullen that the high pH found by Bigwood (1924) using Cullen's (1922) method might be due to the "correction" for epileptic plasma being abnormal; this suggestion, together with the criticism to which Cullen's method of estimating plasma pH has recently been subjected by Austin, Stadie and Robinson (1925) makes it necessary to consider the steps of this method more in detail.

At several steps in the methods of both Cullen and Hastings and Sendroy (1924) apparent or actual changes of pH may occur. It is therefore necessary to consider the relation between—

(1) The actual pH of diluted plasma and the pH estimated colorimetrically.
(2) The pH of diluted plasma (1/21) and of undiluted plasma.
(3) The pH of diluted plasma at 20° C. and 38° C.
(4) The pH of plasma separated from blood at 38° and that separated from the same blood at 20°, as pointed out by Martin and Lepper (1926).

In Hastings and Sendroy's method (3) will not come in.

(1) ACTUAL AND ESTIMATED pH OF DILUTED PLASMA.

The effect of protein on indicators is well known; Hastings and Sendroy (1924), however, consider that in the dilution 1/21 it will be negligible. We have tested this question by comparing the colours of M/30 bicarbonate solutions and M/15 phosphate solutions to which bicarbonate-free plasma or plasma of approximately the same pH as the buffer solutions has been added, with the same buffer solutions to which neutral NaCl solution has been added. Comparisons were made in a comparator with the usual dummy tubes to compensate for the colour of the plasma. The addition of this plasma will make no appreciable difference to the actual reaction of the buffer solutions,

and any difference of colour will be due to the effect of protein on the indicator.

Experiments with Bicarbonate Buffer.

To 10 c.c. of plasma in a round-bottomed flask were added 0·5 c.c. 2N. HCl, the fluid rotated in a thin layer on the wall of the flask to remove CO_2, while CO_2-free air was blown in from time to time; 0·4 c.c. of N/1 NaOH were then added and the reaction of the fluid adjusted colorimetrically with N/10 NaOH. 1 c.c. of this fluid was then added to 20 c.c. of a solution of 0·03N. $NaHCO_3$ and 0·12 N.NaCl containing phenol-sulphone-phthalein in the concentration used in plasma pH estimations. 15 c.c. of this fluid were drawn up into a tube about 1 in. in diameter and of 65 c.c. capacity, with a tap at each end. Into a similar tube were drawn 15 c.c. of the $NaHCO_3$ NaCl mixture, to 20 c.c. of which 1 c.c. of boiled 0·9 per cent. NaCl solution had been added. An air-CO_2 mixture, of such strength as to bring the bicarbonate solution to approximately the pH to which the plasma has been adjusted, was passed through both tubes intermittently; between passages the taps were closed and the fluids well shaken with the gas in the tubes (one minute gas passage, one minute shaking). This was continued for 25 minutes. The tubes were then compared in a comparator, using the usual tubes to compensate for the colour of the plasma. In all cases the plasma tube appeared more acid. By means of standard phosphate solutions the differences were estimated; the results are shown in Table I:

TABLE I.—*The Effect of Plasma Proteins on the Apparent pH of Buffer Solutions.*

Plasma.	Buffer.	Temperature.	Apparent pH of buffer + plasma.	pH of buffer + saline.	Difference.	Protein, gm. per 100 c.c.
Normal, 11	Bicarbonate	Room	7·70	7·75	0·05	7·9
„ 11	„	„	7·375	7·425	0·05	7·9
„ 10	„	„	7·325	7·375 +	0·05 +	—
Nephritic, 12	„	„	7·425 +	7·45	0·025 −	4·6
Epileptic; numerous fits	„	„	7·475	7·50 +	0·025 +	6·9
Epileptic; very numerous fits	„	„	7·425	7·50	0·075	7·9
Normal, 10	Phosphate	38°	7·34	7·40	0·06	—
„ 10	„	20°	7·75	7·80	0·05	—
„ 8	„	38°	7·425	7·50	0·075	7·6
„ 8	„	38°	7·40	7·45	0·05	7·6
„ 8	„	20°	7·68	7·75	0·07	7·6
Nephritic, 12	„	38°	7·375	7·40	0·025	4·6

Experiments with Phosphate Solutions.

The pH of plasma freshly collected under oil was estimated in the usual way and then 1 c.c. of this plasma added without loss of CO_2 to 20 c.c. of a phosphate solution of the same pH. The fluid was covered with melted paraffin, which was allowed to solidify, and the apparent pH of this mixture estimated by comparison with standard phosphate solutions (Table I).

It appears from this table that the protein effect on the indicator is by no means negligible at this dilution, that it will make the pH of the fluid appear 0·05 to 0·07 too low when the protein concentration of the plasma is normal, less when the protein concentration is low. The effect is the same at 20° C. and 38° C.

(2) pH OF DILUTED AND UNDILUTED PLASMA.

Hastings and Sendroy (1924) show that the reaction of a bicarbonate solution did not change on diluting with 0·9 per cent. NaCl. However, this does not appear to be the case when plasma is diluted. In practically all the cases in which the colorimetric pH of diluted plasma and electrometric or gasometric pH of undiluted plasma have been estimated at the same temperature (Hastings and Sendroy, 1924; Cullen and Robinson, 1925; Austin, Stadie, and Robinson, 1925), the colorimetric pH has been the same, more alkaline, or at most only slightly more acid. Now since the effect of protein on the indicator would, as we have shown, make the colorimetric pH some 0·05 less than the actual pH, we must infer that the actual pH is raised on dilution. Such a change of pH might well be expected when we consider the effect of protein on the activity of ions found by Frisch, Pauli and Valko (1925), also the difference found by Warburg (1922) in the apparent first dissociation constant of H_2CO_3 in solutions in which the sodium and potassium are the principal kations, but without fuller knowledge of the effect of protein on the activity of ions we cannot calculate this change.

We have compared the electrometric pH at 38° of CO_2-free plasma (undiluted) with the colorimetric (diluted 1/21) in 3 cases (Table II). In these the difference found would be accounted for by the protein indicator effect alone. These experiments suggest, therefore, that this dilution effect does not occur with protein solutions alone, but the number of experiments, which was limited owing to an accident to our potentiometer during the general strike and the inability of one of us to continue this work later, is insufficient to justify a final conclusion.

TABLE II.—*Comparison of Electrometric and Colorimetric pH of Bicarbonate-free Plasma at* 38°.

Plasma.	A. Electrometric pH, 38° C.	B. Colorimetric pH, 38° C.	Difference, A—B.	Protein, gm. per 100 c.c.
Mixed diabetic . .	7·84	7·82	0·02	6·8
Nephritic, 13 . . .	7·24	7·19	0·05	4·1
„ 14 . . .	7·37	7·34	0·03	5·1

(3) pH OF PLASMA AT 20° C. AND 38° C.

Stadie, Austin and Robinson (1925) give an equation connecting this temperature correction ($=_{t\text{-}t'}\Delta$ pH) with the reaction, protein and bicarbonate concentration of the plasma,

$$({}_{t\text{-}t'}\Delta pH - {}_{t\text{-}t'}\Delta pI)\ \beta.\ [Pr.] = \left(\frac{k'_{t'}}{k'_{t'} + [H^+]_{t'}} - \frac{k'_t}{k_t' + [H^+]_t}\right).\quad [CO_2] \longrightarrow I;$$

assuming that the value of $[\beta.\ Pr.]$ found by Van Slyke, Wu, and MacLean (1923) for horse plasma is correct for human plasma then $\beta.\ [Pr.] = 5$, when the protein concentration = 7·4 per cent. The value of ${}_{t-t'}\Delta$ pI can be found by estimating colorimetrically at temperatures t and t' the pH of the bicarbonate-free diluted plasma obtained in Van Slyke's (1922) titrimetric method of estimating plasma bicarbonate. Since we have found that the protein effect on the indicator is unchanged with temperature, and since the dilution is unchanged, the difference thus found should be the actual change of pH with temperature. The average value found for ${}_{38°\text{-}20°}\Delta$ pI in normal and epileptic plasma was − 0·35. This makes ${}_{38°\text{-}20°}\Delta$ pH in plasma about − 0·26. The average value we found in normals was 0·247, in epileptics 0·233. Variation from these values occurred unaccounted for by variation in the terms of the equation; these would be partly accounted for by errors in colorimetric reading. In two nephritic plasmata cases, 13 and 14, the difference between the pH of the bicarbonate-free plasma at 38° and 20° was only 0·22. This would account for the low values found in the cases for the change of pH of the whole plasma.

In estimating plasma pH by the method of Hastings and Sendroy this temperature correction will not come in; in using Cullen's method the change of the temperature correction owing to low protein will be partly neutralized by the reduction of the protein effect on the indicator (so that in case 12, Table III, the resultant error would only be 0·05), and the change due to low bicarbonate may be partly neutralized by the effect of an associated low pH. These mutually neutralizing effects account for the constant value for Cullen's total correction found by Marrack and Smith (1924) in abnormal plasmata.

(4) pH OF PLASMA SEPARATED AT 20° C. AND AT 38° C.

Stadie, Austin and Robinson (1925) found that the pH of true plasma of dog's blood separated at 38° was about 0·32 lower than that separated at 20°. This would be expected from equation I applied to whole blood, for the effect of the other factors proves negligible in comparison with that of hæmoglobin, so that the temperature coefficient of blood is the same as that of hæmoglobin. Now if human plasma is separated at 20° and warmed to 38° the pH will only fall about 0·26. We should therefore expect a difference of pH between plasma separated from blood at 38° and that separated at 20° when these are compared at the same temperature. We have compared such

plasmata from samples of blood taken simultaneously from the same subject. Out of 10 normal cases the plasma separated at 20° was more alkaline in 8, and out of 10 epileptics more alkaline in 8; and the average difference in pH was 0·03 in normal, and 0·035 in epileptics. This difference is therefore not large, and is probably smaller in practice when a good volume of blood is taken and centrifuged at once, as in this case the separation will be complete before the blood has cooled.

It appears from these considerations that the total error in these colorimetric methods, apart from that due to the change of actual pH produced by dilution, should not exceed about 0·05. Now when we consider the total error we find that, comparing the pH at 38° measured electrometrically or gasometrically with the colorimetric pH at 38°, Hastings and Sendroy (1924) found differences up to 0·04 in normal men and horses; comparing with the colorimetric at 20° and using a constant correction, Cullen (1922) found differences in normal men up to 0·04, in horses up to 0·06, in dogs up to 0·03; Cullen and Robinson (1925) found differences up to 0·12 in abnormal human plasma, and Marrack and Smith (1924) differences up to 0·04 in plasmata specially selected for abnormality. In this connection we may point out that Marrack and Smith calculated the pH of plasma from $[CO_2]$ and $[HCO_3]$ using the value for $pK' = 6·14$, as given by Austin *et al.* (1922); if in their experiments the value 6·096 found by Cullen (1925) is used, values for the total correction (difference between colorimetric pH at 20° and actual pH at 38°) ranging from 0·25 to 0·31 are obtained; these agree well with Cullen's (1925) figures for pathological sera 0·24 to 0·34.

On the other hand, Austin, Stadie and Robinson found differences between the gasometric and colorimetric pH both at 38°, in dogs up to 0·17 with an average 0·08 and up to 0·20 in human patients in whom no great abnormality would be expected.

It appears probable that some other circumstances peculiar to their work must have led to their exceptional results.

We conclude that the colorimetric method of estimating plasma pH gives results accurate within 0·05 with normal plasmata, and within 0·12 with abnormal plasmata.

It must be remembered at the same time that there may be great variations in the venosity of blood obtained from the veins—the variation of oxygen saturation is made obvious by the colour of the blood. This variation again will introduce appreciable variations in the pH found. There is also always a possibility that some degree of hyperpnœa may occur in patients before and during bleeding, leading to a higher plasma pH than is usual with them.

COMPARISON OF COLORIMETRIC pH OF PLASMA IN NORMAL AND EPILEPTIC SUBJECTS.

Our normals were healthy laboratory workers. The epileptics were in-patients in the wards of the Medical Unit or out-patients of the London Hospital, or out-patients at Maida Vale Hospital. Bleeding was done without stasis or contraction of arm muscles, with a large needle into 20 c.c. centrifuge

tubes as described by Marrack (1923); 10 c.c. of blood were allowed to run off before collection for pH was begun. When the effect of cooling was studied the needle was attached to a Y tube, and blood collected simultaneously in two centrifuge tubes containing potassium oxalate and fluoride to check the formation of acid. The tubes were re-warmed in a water-bath at 38° after filling, then one was well lagged with cotton-wool and rapidly contrifuged. Preliminary experiments showed that it would not cool more than 3° in this process. Plasma was then drawn off for pH estimation in the usual way. The second tube was then removed from the water-bath (both were thus kept warm for the same time), shaken up and cooled for at least 10 minutes in a water-bath at 20°, with occasional shaking. It was then centrifuged and plasma pH estimated. The bloods from patients at Maida Vale were collected in tubes containing oxalate fluoride, the tubes were immersed in ice-cold water and taken to the London Hospital in about half-an-hour. They were then warmed to 38° before the plasma was separated. The readings at 38° were made with Hastings and Sendroy's (1925) bicolor standards, those at 20° with phosphate standards. The bicolor standards agreed with the phosphate standards at 38°, allowing 0·03 for the change in pH of phosphates at that temperature. The phosphate standards were checked electrometrically.

TABLE III.—*Colorimetric pH of Plasmata at* 38° *and at* 20°; *the Plasma has been Separated from Whole Blood at* 38° *and at* 20°. *Control Cases.*

	Plasma separated at 38°. A.			Plasma separated at 20°. B.			B—A.	HCO_3; M × 10^3.	Protein, gm. per 100 c.c.	Remarks.
	38°.	20°.	20°–38°.	38°.	20°.	20°–38°.				
Normal, 1 .	7·42	7·67	0·25	7·45	7·71	0·26	0·03	29·5	—	
„ 2 .	7·45	7·70	0·25	7·51	7·76	0·25	0·06	28	7·2	
„ 3 .	7·50	7·70	0·20	7·47	7·69	0·22	−0·03	24	7·6	
„ 4 .	7·42	7·68	0·26	7·44	7·68	0·24	0·02	29	6·9	
„ 5 .	7·44	7·68	0·24	7·50	7·72	0·22	0·06	27·5	7·2	
„ 6 .	7·47	7·725	0·255	7·51	7·80	0·29	0·04	27·5	7·8	
„ 7 .	7·40	7·65	0·25	7·40	7·65	0·25	0·00	25	7·1	
„ 7 .	7·31	7·54	0·23	7·34	7·58	0·24	0·03	27	—	
„ 8 .	7·40	7·62	0·22	7·42	7·63	0·21	0·02	27	7·6	
„ 8 .	7·38	7·68	0·30	7·42	7·71	0·29	0·04	—	—	
„ 9 .	7·42	7·67	0·25	—	—	—	—	—	—	
„ 10 .	7·35	7·61	0.26	7·34	7·60	0·26	−0·01	—	—	
Average . .	7·413	7·660	0·247	7·437	7·685	0·248	0·024	27	7·3	
Nephritis, 11 .	7·24	7·51	0·27	7·23	7·50	0·27	−0·01	8·5	7·2	Plasma P, 0·011 %.
„ 12 .	—	—	—	7·50	7·67	0·17	—	22·5	4·6	„ „ 0·0047 %.
„ 13 .	7·24	7·43	0·19	—	—	—	—	8·0	4·1	„ „ 0·0125 %.
„ 14 .	7·50	7·72	0·22	7·52	7·76	0·24	0·02	29	5·1	„ „ 0·0053 %.
Diabetes, 15 .	7·44	7·68	0·24	—	—	—	—	26	—	
Meningitis, 16	7·50	7·72	0·22	—	—	—	—	28	6·9	
Tetany, 17 .	7·40	7·65	0·25	7·41	7·64	0·23	0·01	22	8·6	
Jacksonian epilepsy, 18	7·33	7·56	0·22	—	—	—	—	—	—	

The plasma pH found by us in epileptics (Tables IV and V) is the same as that found in normal subjects. Our normal range is higher than is usually given—Cullen and Robinson (1923) 7·30–7·40, Bigwood (1924) 7·33–7·39, Myers and Booher (1924) 7·34–7·43. This may be partly due to the free flow of blood we have obtained and the discarding of the first 10 c.c. In any case our estimations with epileptic and normal subjects are done similarly and the results are comparable. We therefore differ from Bigwood in the higher range found in normals. The fall of pH found by Bigwood on drug treatment may well have been due to depression of the respiratory centre. The same effect may have occurred in some of our cases under treatment, but a high plasma pH cannot be considered to play an essential part in producing fits, since we have found normal pH in cases having numerous fits.

TABLE IV.—*Colorimetric pH of Plasmata at* 38° *and at* 20°; *the Plasma has been separated from Whole Blood at* 38° *and at* 20°. *Epileptics.*

		Plasma separated at 38°. A.			Plasma separated at 20°. B.			B—A.	HCO_3, M × 10^3.	Protein, gm. per 100 c.c.	Frequency of fits.
		38°.	20°.	20°–38°.	38°.	20°.	20°–38°.				
1	17 . 3 . 26	7·50	7·725	0·225	7·49	7·72	0·23	−0·01	27·5	7·0	2 fits this day.
2	29 . 3 . 26	7·43	7·66	0·23	—	—	—	—	27·5	—	2 a week.
1	17 . 3 . 26	7·42	7·65	0·23	—	—	—	—	27	—	Fit-free period.
3	29 . 3 . 26	7·42	7·65	0·23	—	—	—	—	26·5	—	2 to 5 a week.
4	8 . 3 . 26	7·42	7·64	0·22	7·41	7·64	0·23	−0·01	26	—	Very infrequent.
5	9 . 3 . 26	7·42	7·66	0·24	—	—	—	—	—	—	3 a day.
6	15 . 2 . 26	7·41	7·61	0·20	—	—	—	—	—	—	2 a week.
7	5 . 2 . 26	7·405	7·60	0·195	—	—	—	—	—	—	Every 2 weeks.
8	2 . 3 . 26	7·40	7·63	0·23	7·41	7·63	0·21	0·01	—	—	Several a week.
9	4 . 3 . 26	7·40	7·63	0·23	7·44	7·68	0·24	0·04	—	—	Over 1 a week.
10	31 . 1 . 26	7·40	7·60	0·20	—	—	—	—	—	—	4 fits this day.
11	29 . 3 . 26	7·40	7·59	0·19	—	—	—	—	26·5	—	2 a month.
1	21 . 1 . 26	7·38	7·56	0·18	—	—	—	—	—	—	2 to 6 a day.
12	3 . 5 . 26	7·37	7·65	0·28	—	—	—	—	29	6·9	3 a week.
13	26 . 2 . 26	7·36	—	—	7·41	—	—	0·05	—	—	1 a month.
10	17 . 2 . 26	7·36	7·61	0·25	7·43	7·68	0·23	0·07	26	—	2 or more a day.
14	5 . 3 . 26	7·36	7·61	0·25	7·40	7·63	0·23	0·04	—	—	2 a week.
15	10 . 3 . 26	7·36	7·60	0·24	—	—	—	—	—	—	2 to 3 a day.
16	27 . 3 . 26	7·36	7·60	0·24	—	—	—	—	26·5	—	2 a month.
17	3 . 4 . 26	7·36	7·65	0·29	—	—	—	—	29·5	6·7	6 a month.
18	1 . 3 . 26	7·35	7·59	0·24	7·40	7·62	0·22	0·05	—	—	4 a week.
1	14 . 4 . 26	7·35	7·61	0·26	—	—	—	—	27	—	Fit-free period.
19	3 . 4 . 26	7·35	7·59	0·24	—	—	—	—	28·5	—	2 to 6 a month.
20	18 . 1 . 26	7·34	7·56	0·22	—	—	—	—	—	—	Fit-free period.
21	1 . 2 . 26	7·34	7·58	0·24	—	—	—	—	—	—	3 a week.
22	8 . 2 . 26	7·34	7·57	0·23	—	—	—	—	—	—	1 a week.
23	11 . 2 . 26	7·34	7·58	0·24	—	—	—	—	—	—	Infrequent.
24	10 . 3 . 26	7·33	7·55	0·22	7·36	7·59	0·23	0·03	—	—	None recently.
25	3 . 3 . 26	7·30	7·54	0·24	7·38	7·62	0·24	0·08	—	—	2 a week.
26	3 . 4 . 26	7·30	7·59	0·29	—	—	—	—	30	6·2	2 to 6 a month.

TABLE V.—*pH of Plasma of Epileptics by Cullen's Original Method.*

		pH.	HCO_3, $M \times 10^3$.	Protein, gm. per 100 c.c.	
27	20 . 4 . 25	7·47	26	—	Recovery from status epilepticus.
28	31 . 12. 24	7·35	27	—	Frequent slight fits daily.
29	21 . 1 . 25	7·42	22·5	8·3	About 2 a week.
	2 . 2 . 25	7·32	30	—	" " "
	6 . 2 . 25	7·37	27·5	—	" " "
30	27 . 2 . 25	7·42	32	—	About 3 a week.
	2 . 3 . 25	7·42	30	—	" " "
	10 . 3 . 25	7·52	15	—	" " " Starving.
	16 . 3 . 25	7·42	18	—	" " " "
31	9 . 2 . 25	7·31	30·5	—	2 a week.
32	1 . 5 . 25	7·36	21	—	Frequent slight fits daily.
	7 . 5 . 25	7·35	27	—	" " "
	16 . 5 . 25	7·45	25·5	—	" " " Starving.
	21 . 5 . 25	7·45	21	—	" " " "
33	11 . 6 . 25	7·44	26	—	About 6 a week.
	13 . 6 . 25	7·12	25	—	" " " During fit.
	14 . 6 . 25	7·38	15	—	" " " Starving.
34	6 . 4 . 25	7·42	23	—	Fit-free period.
	15 . 4 . 25	7·37	26	—	" "
	22 . 4 . 25	7·37	17	8·5	" " Starving.

The range of pH that we have found is very similar to that found with Cullen's method by Dautrebande (1926) using arterial blood. We do not, however, agree with him in his calculations of the plasma pH by the Henderson-Hasselbalch equation. Since the total CO_2 found in the blood of most of his patients is less than that which the blood contained when exposed to a gas mixture containing CO_2 at the tension which he found in alveolar air, we infer that the CO_2 tension in the patient's blood at the time of bleeding was less than that in the alveolar air samples taken 10 minutes before; we do not think his suggestion of a hypothetical substance, which combines with difficulty with CO_2 *in vivo*, satisfactory. We should therefore use as CO_2 tension, in calculating the plasma pH, the abscissa of the point on the blood dissociation curve whose ordinate is the total CO_2 content of the actual sample of blood under consideration. The average of the values thus calculated agrees well with the average of the values obtained by Cullen's method. The same criticism applies to Dautrebande's calculation of the pH of the blood, and he appears to have used 6·1 as his value of pK′ for blood; recalculating from his figures we again get a good agreement on the average with the estimations by Cullen's method.

The colorimetric method therefore gives no evidence of abnormal blood reaction in epileptics, nor is there any evidence of any abnormality which would make colorimetric estimations incorrect; the temperature correction is not abnormally large.

We may add that we have found no evidence of the abnormal ammonia

excretion which Norvig (1924) regards as evidence of disturbed neutrality regulation in epileptics.

SUMMARY.

1. The sources of error in colorimetric estimation of plasma pH are discussed. It is not considered that the error exceeds 0·05 with normal plasma nor 0·12 with abnormal plasma.

2. The effect of the temperature at which plasma is separated from blood on the pH of the plasma has been studied.

3. The hydrogen ion concentration of the venous blood of epileptics has been found to lie within the normal range.

The expenses of this work have been defrayed in part by the London Hospital fund for the study of epileptic states.

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ON THE EFFECT OF VARIATIONS OF THE SALT CONTENT OF BLOOD ON ITS BACTERICIDAL POWER *IN VITRO* AND *IN VIVO*.

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Received for publication July 15th, 1926.

THE author (1924) has described the action of chemical antiseptics on the growth of bacteria in blood, and has shown that with practically every antiseptic tested there were, as regards bacterial growth, three zones of concentration of the chemical. These were: (*a*) An "indifferent" zone in which the chemical was too weak to have any effect on the bacterial growth; (*b*) an "antibactericidal" zone, in which the concentration was sufficient to inhibit or destroy the normal bactericidal power of blood without inhibiting the bacteria so that an increased microbic growth results; and (*c*) an "antiseptic" zone, in which the strength was sufficient to inhibit or destroy the bacteria as well as the leucocytes of the blood. These results were obtained not only with chemicals which are used as antiseptics, but with a great variety of substances, and in this article the results which have been obtained with common salt are detailed.

EFFECT OF INCREASING THE SALT CONTENT ON THE BACTERICIDAL POWER OF BLOOD.

This was investigated by the slide-cell method (Wright, 1923). Blood mixed with salt solution in various concentrations was infected with a known number of bacteria placed in slide-cells, incubated, and the number of colonies which developed was enumerated. Counts were made after 24 hours, and frequently also after longer intervals, but it was found that in 24 hours results were obtained which did not materially change after longer periods of incubation, so the figures given represent in all cases the number of colonies developing after 24 hours.

A rough experiment was done by making a progressive series of dilutions of saturated salt solution in 0·85 per cent. saline, adding to one volume of each of those dilutions one volume of infected blood, and incubating the mixtures in slide-cells. The result obtained was as follows:

Final concentration of salt in blood (taking blood to be equal to 0·85% salt)	13%	7%	4%	2·4%	1·6%	1·2%	1·1%	0·97%
Number of colonies developing	0	68	70	78	74	70	31	3

Number of microbes implanted in each cell (estimated by plating on agar) . . . . . 98 staphylococci.

This experiment shows that with sodium chloride, just as with chemical antiseptics, there are the three zones of concentration—the indifferent zone, represented by concentrations less than 1·1 per cent., the antibactericidal zone, between 1·1 and 7 per cent., where there is an increased growth of staphylococci, and the antiseptic zone, represented by the semi-saturated salt solution where the growth of the cocci is inhibited.

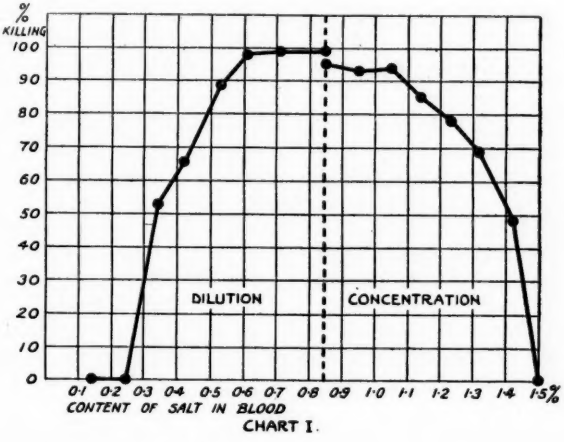

CHART I.

As in this experiment the gradation of dosage of salt was rather coarse, another experiment was undertaken using a closer range of concentrations. In this case 10 volumes of blood were used to one of salt solution, otherwise the technique was identical. The results are shown on Chart 1, and it will be seen from this that there is complete inhibition of the normal bactericidal power of the blood when the salt concentration reaches 1·5 per cent., so that all the microbes which are implanted in the blood grow out.

EFFECT OF REDUCING THE SALT CONTENT OF BLOOD ON ITS BACTERICIDAL POWER.

This was tested by adding to blood varying amounts of distilled water. To each 50 c.mm. volume of blood, or blood thus diluted, a suitable implant of staphylococci was added, and the mixtures were incubated in slide-cells.

To ascertain the effect which mere dilution had on the results, a similar series of dilutions were made of the blood in normal salt solution and treated in the same manner. The results are shown in Table I:

TABLE I.—*Effect of Diluting the Blood on its Bactericidal Power.*

Dilution of blood.	Percentage of staphylococci killed: When water was diluent.	When normal salt solution was diluent.
Undiluted	99	99
5 to 1	99	99
5 to 2	98	98
5 to 3	89	97
1 to 1	66	93
2 to 3	53	92
2 to 5	0	94

Implant of staphylococci per slide-cell 300.

From this table it will be seen that when the dilution with water is such that the salt content is reduced to 0·5 per cent. there is a certain amount of inhibition of the normal bactericidal power, and that when it is reduced to 0·24 per cent. every microbe implanted grows out. The fact that it is not mere dilution which produces this result is shown by the results when 0·85 per cent. salt solution is used as the diluent, when there is little reduction of the bactericidal power of the blood in any of the dilutions.

The results obtained by reducing the salt content of blood have been combined with those following the addition of hypertonic solutions in Chart 1. The figures there plotted in a curve show in graphic form the percentage of the implant killed by blood, the salt content of which has been increased or diminished.

It has been demonstrated that the power of blood to kill staphylococci is a function of the leucocytes. Certain observations have therefore been made to see what is the effect of altering the salt content of blood on some of the other functions of leucocytes, n mely emigration and phagocytosis.

EFFECT OF THE SALT CONTENT OF BLOOD ON LEUCOCYTIC EMIGRATION.

It is well known that if a drop of blood is placed in a glass slide the leucocytes will emigrate from the clot and adhere to the slide. The effect of anything which injures the leucocytes is to inhibit, partially or completely, this emigration.

Blood was mixed with one-tenth of its volume of various concentrations of salt solution or with various quantities of water, and of the mixtures 25 c.mm. volumes were placed in small cells, 6 mm. in diameter, on a microscope slide, and incubated in a moist chamber for one hour. The blood-clots were then thrown off and the slide washed gently to remove the serum and red corpuscles,

after which the slide was stained, and the number of emigrated leucocytes enumerated. The margin of error in such counts is considerable, but the results obtained which are plotted on Chart 2 show that the emigration in relation to the salt content follows roughly the same curve as does the bactericidal power of the blood (Chart 1).

EFFECT OF THE SALT CONTENT OF BLOOD ON PHAGOCYTOSIS.

Defibrinated blood was mixed with one-tenth of its volume of hypertonic salt solutions of various strengths, or was diluted in varying degrees with water, and of these mixtures two volumes were added to one volume of a

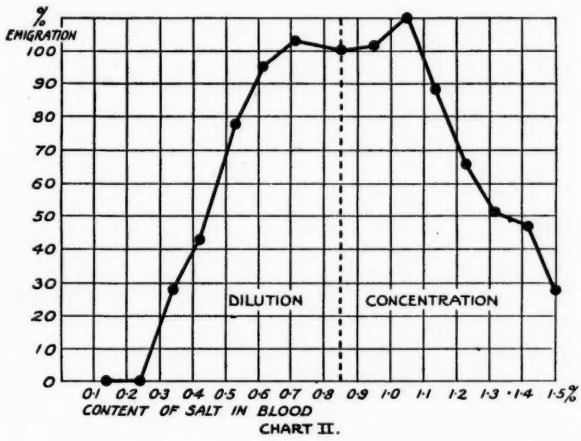

CHART II.

staphylococcus suspension (in a fluid of known salt content) and incubated for 15 minutes, when films were made and the number of microbes ingested was estimated in the usual way.

The results obtained are set out in Chart 3, and it will be seen from a comparison of this with Charts 1 and 2 that the effect of altering the salt content of the blood has the same effect on phagocytosis as it has on leucocytic emigration and on the bactericidal power of the blood.

It appears, therefore, that the leucocytes can only function when the salt content of the blood is within a certain range, and they are destroyed when the salt content falls below 0·25 per cent., or is raised above 1·5 per cent.

The inhibitory action of hypertonic salt on the bactericidal action of the blood can be demonstrated not only with staphylococci, but also with microbes such as *Streptococcus viridans*, *Streptococcus pyogenes*, or *B. coli*. Even when the salt concentration is as high as 2·9 per cent. as it is in an equal mixture of 5 per cent. salt solution and blood, there appears to be no lethal action on these bacteria and there is very little inhibition of their growth. This concentration

of salt inhibits very materially coagulation of the blood, so that after 6 or 7 hours only a loose clot has formed. This anti-coagulant power combined with the lethal effect on the leucocytes and the absence of any destructive action on bacteria suggested the probability that 5 per cent. salt solution might be a very suitable fluid to use for blood culture and this has been found to be the case. My colleague, Dr. Colebrook, will shortly publish a communication on this subject.

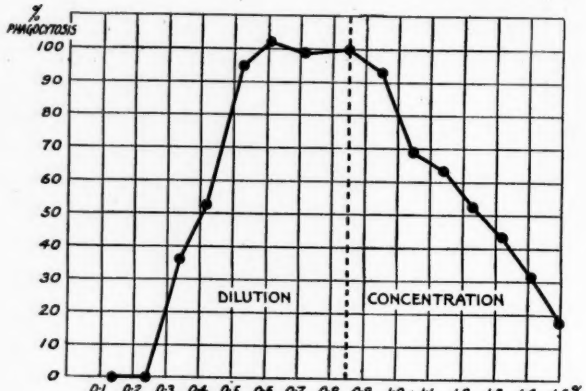

CHART III.

EFFECT OF CONCENTRATED SALT SOLUTION INJECTED INTRAVENOUSLY ON THE BACTERICIDAL POWER OF THE BLOOD.

The fact that a comparatively small increase in the salt concentration of blood *in vitro* has a very considerable influence on the bactericidal power of the leucocytes led to the investigation of the results on intravenous injection of hypertonic salt solution. Such solutions have been used therapeutically in various conditions, but no attention has been paid to the question as to whether they influence the bactericidal power of the blood of the individual injected.

Several rabbits were taken, and various amounts of a 10 per cent. solution of sodium chloride were injected intravenously. Blood specimens were taken before and at intervals after the injection, the blood was defibrinated, and its bactericidal power examined by the slide-cell method.

The first rabbit received a very large dose of the hypertonic salt solution, 20 c.c. of 10 per cent., which was sufficient to raise the salt content of its blood by about 1·5 per cent. For about a minute after the injection the rabbit was distressed and had a short convulsive attack, after which it appeared to be normal. Blood taken 2 minutes after the injection showed the red corpuscles definitely crenated.

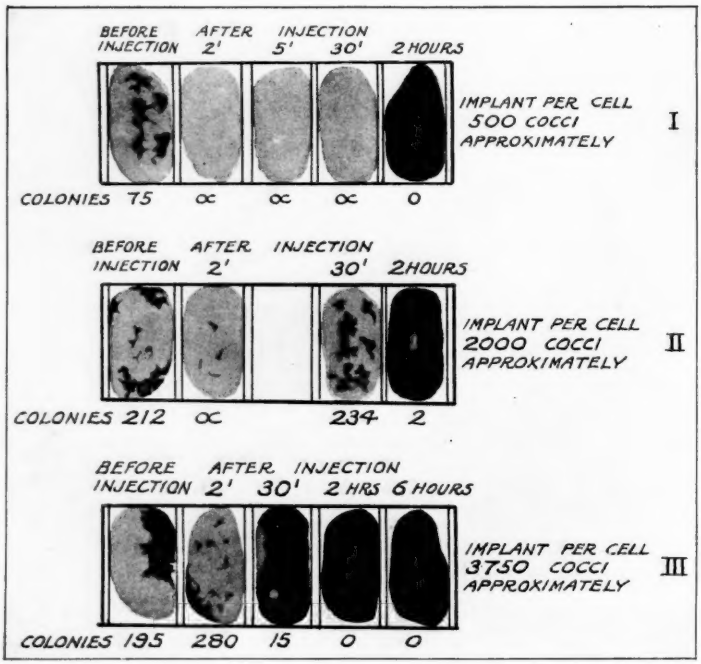

FIG. 1.

I = Rabbit (weight 1720 gm.) injected with 20 c.c. 10 per cent. salt.
II = Rabbit (weight 2520 gm.) injected with 5 c.c. 10 per cent. salt.
III = Rabbit (weight 2600 gm.) injected with 3 c.c. 10 per cent. salt.

The blood before the injection was able to kill 73 per cent. of a small implant of staphylococci (300 per c.c.) In specimens taken 2 and 5 minutes after the injection the bactericidal power of the blood had been completely lost, which is in accord with the *in vitro* results.

Half an hour after the injection the bactericidal power of the blood was only slightly worse than it was originally, and two hours after it had improved to such an extent that the whole of the implant had been killed.

This last result with the blood taken 2 hours after injection was unexpected, and it pointed to possibilities in the use of hypertonic salt injection for therapeutic purposes in bacterial infections. Smaller amounts of the salt solution were injected to see if the same increase in the bactericidal power could be obtained without the preliminary negative phase. Five c.c. of 10 per cent. salt solution were injected into a rabbit's vein, increasing the salt concentration of its blood by about 0·25 per cent., and here there was only a comparatively slight reduction in the bactericidal power of the blood immediately after and 30 minutes after the injection, but after 2 hours the power had increased so that the whole of an implant of 1100 staphylococci per c.c. were destroyed. Another rabbit was injected with 3 c.c., increasing the salt concentration of the blood by about 0·15 per cent., and here in the specimens taken immediately after the injection there was no diminution of the bactericidal power, in the specimen taken 30 minutes after injection there was a considerable increase, while in the specimens taken 2 and 6 hours after the whole of the implant of 3860 staphylococci were killed (Table II).

This result has been confirmed on other animals, and there seems no doubt that a small injection of hypertonic salt solution is followed by a great increase in the bactericidal power of the blood, which is evident after about half an hour and which persists for some hours.

TABLE II.—*Effect of Intravenous Injection of Hypertonic Salt Solutions into Rabbits on the Bactericidal Power of the Blood.*

Weight of rabbit in gm.	Amount of 10% salt solution injected c.c.	Approximate increase in salt content of blood.	Percentage killing of staphylococci in blood taken —						Implant of staphylococci per c.c.
			Before injection.	After injection.					
				2 min.	5 min.	30 min.	2 hrs.	6 hrs.	
1720	20	1·5%	73	0	0	60	100	—	300
2520	5	0·25%	66	36	—	57	100	—	1100
2600	3	0·15%	75	72	—	92	100	100	3860

The alterations in the bactericidal power of blood following the intravenous injection of hypertonic salt solutions are shown in a very striking manner when a hæmolytic streptococcus is used as the test organism. The results obtained in this way are illustrated in Fig. 1 where the lighter shading represents the areas of hæmolysis resulting from bacterial growth and the deeper shading represents the non-hæmolysed blood.

It will be seen at a glance that the larger amounts of salt temporarily reduced the bactericidal power, thus allowing greater growth of streptococcus and consequently more hæmolysis, but that after 2 hours with all the rabbits there was almost complete killing of the streptococci.

In view of these results in rabbits an injection of hypertonic salt solution was made into a patient, and observations were made on the blood before and after the injection.

The patient was a boy suffering from phthisis. He received an intravenous injection of 50 c.c. of 10 per cent. salt solution, which when diluted with his whole blood raised the salt concentration by only about 0·02 per cent. He suffered no inconvenience from the injection.

The bactericidal power of the blood was tested by incubating in slide-cells 2·5 c.mm. of a suitable dilution of staphylococcus with 50 c.mm. of defibrinated blood, with the following results:

Time blood taken.	Number of colonies.	Percentage killing.
Before injection	135	82
2 minutes after injection . . .	145	74
1 hour after injection	74	88
3½ hours after injection . . .	6	99
22 „ „ „ . . .	25	95

A similar result was obtained with hæmolytic streptococci, and although in this case accurate counts could not be obtained, the specimen taken 3½ hours after injection showed practically no hæmolysis, whereas the specimen taken before injection was completely hæmolysed, and several hundred colonies of streptococci had grown out.

These results were confirmed on another patient, where the amount of salt injected when diluted with the patient's blood would only raise the salt concentration by 0·01 per cent. There was again a marked increase in the bactericidal power of the blood three hours after the injection.

Some observations were made with a view to ascertaining, if possible, the reason for this increase in the bactericidal power of the whole blood. It was found that the serum had no bactericidal power to staphylococcus at any stage of the experiment. The number of leucocytes did not vary much, and what small variations there were did not correspond with the killing power of the blood. There were no significant variations in the opsonic power of the serum or in the efficiency of the leucocytes to ingest bacteria. The explanation of the increased bactericidal power of the blood was not therefore forthcoming from the experiments.

DISCUSSION.

It has been shown in the earlier portion of the paper that leucocytes only function efficiently within certain limits of salt concentration, and that when

the salt content falls below or rises above these limits the leucocytes do not emigrate and do not phagocyte or destroy bacteria.

It has been shown also that when a large amount of salt is injected into an animal so that the salt content of the blood is materially raised, the normal bactericidal power is temporarily lost, but that after a few hours it is greatly enhanced. When smaller doses are injected the preliminary fall in the bactericidal power is avoided, but the subsequent rise is marked, and in man the injection of an amount of salt so little that when diluted with the blood it would only increase the salt content by 0·01 per cent., causes after 3 hours a great rise in the bactericidal power of the blood. So far the experiments have thrown little light on the reason for this astonishing rise in the bactericidal power, but it is hoped that further work may be more profitable in this direction.

The intravenous injection of hypertonic salt solution has been used in cholera and other diseases with a view to increasing the blood volume, but the results detailed above show that such injections may produce benefit, not only by virtue of their fluid content, but also by conferring on the patient's blood much greater powers of destroying the infecting bacteria.

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A SIMPLE METHOD OF REMOVING LEUCOCYTES FROM BLOOD.

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Received for publication July 15th, 1926.

It has been a common observation that leucocytes adhere with great readiness to strands of fibrin, cotton-wool, glass or similar substances when blood is incubated in contact with these. Sir Almroth Wright (1926) has described a method whereby blood passes through a single sheet of filter-paper, and has shown that large numbers of the leucocytes adhere to the upper layers of the filter. This method, however, was impracticable when it was desired to obtain any quantity of deleucocyted blood, and it was thought that it might be possible to filter leucocytes out of blood by passing the blood

through a cotton-wool plug. After various trials it was found that, by a very simple process, practically the whole of the leucocytes could be removed from blood. All that is necessary is to pass blood under pressure through a tightly-packed cotton-wool plug. The exact nature of the apparatus will depend to some extent on the amount of blood from which it is desired to remove the leucocytes, but in my experiments where only about 1 or 2 cubic centimetres of blood were used the apparatus shown in Fig. 1 was found to be very convenient.

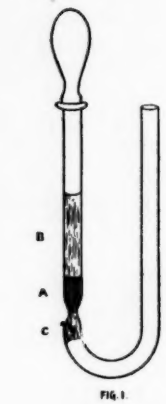

FIG. 1.

A constriction is made in a piece of 6 mm. glass tubing, and below the constriction the tube is bent as shown in the diagram. Some cotton-wool is now introduced into the constricted limb of the tube and pressed down on to the constriction as tightly as possible to make, when compressed, a pad about a quarter of an inch thick. (A convenient instrument for applying the pressure is a small cork-borer.) Blood is now placed in the tube above the cotton-wool, and pressure is applied by means of a rubber teat so that the blood is driven through into the bend of the tube. If the cotton-wool has been very tightly packed then a single passage through the plug will be sufficient to remove practically all the leucocytes, but it is safer to pass it through three times. This can easily be done in the following manner:

(1) Force the blood through the cotton-wool into the bent portion of the tube.
(2) Put the teat on the other limb and force the blood back through the plug.
(3) Replace the teat in its original position and repeat the first procedure.

It is obviously important that the blood should finish in the bend of the tube, where it is not contaminated by the wash of the original blood which has not been through the filter.

If larger quantities of blood are being filtered then a larger tube would be used and a thicker filter. In such cases pressure can be conveniently applied by means of a 10 or 20 c.c. record syringe.

That such procedure actually removes the leucocytes from blood is shown by the following counts made before and after filtering normal human blood. The counts were made in the ordinary way in a hæmocytometer cell, and the figures given represent the numbers of leucocytes per field, using a half-inch objective:

	First experiment.	Second experiment.
Before filtering . . .	148	153
After ,, once . .	15	8
,, ,, twice . .	3	5

Films of the filtered blood were stained, and it was found that all the white corpuscles present were lymphocytes.

After filtration there was no diminution in the numbers of red corpuscles.

While cotton-wool is the most convenient substance of which to make the filter, other substances may be used. Teased filter-paper compressed in the way described is very efficient, but unless great care is exercised the filter may be packed so tightly that blood will only pass through under greater pressure than can conveniently be applied. Glass-wool did not remove the leucocytes quite so easily as cotton-wool, and at the same time the filters were more difficult to make.

EFFECT OF FILTERING BLOOD ON ITS ANTIBACTERIAL POWERS.

It was desired to use deleucocyted blood as a diluent for blood in certain tests in connection with the antibacterial powers of leucocytes, so it was first necessary to investigate the effect of filtering blood through cotton-wool on its antibacterial powers. It was thought that possibly in its passage through the cotton-wool some of the antibacterial substances present in the serum had attached themselves to the cotton-fibres in the same way that they become attached to granules of kaolin, starch or other similar substances.

Opsonic power.—This was tested by comparing the serum from unfiltered and filtered blood, using the same washed corpuscles and staphylococcal suspension in each case.

The result obtained was as follows:

	No. of cocci per 100 leucocytes.
Serum from unfiltered blood . . .	4·22
„ filtered „ . . .	4·29

It is clear, therefore, that filtering through cotton-wool has no influence on the opsonic power of serum.

Agglutinating power.—The agglutinating power of the serum to staphylococci was found to be the same whether the serum was from filtered or unfiltered blood.

Bactericidal power of filtered blood to staphylococcus.—This power, which has been shown to be a function of the leucocytes, was found to be completely abolished.

RELATION OF THE NUMBER OF LEUCOCYTES IN THE BLOOD TO THE AMOUNT OF PHAGOCYTOSIS.

It has already been shown that the process of filtering off the leucocytes from blood has no influence on the opsonic power of the serum. The dilution of blood with deleucocyted blood would, therefore, leave the opsonic power unaltered while altering the number of leucocytes. Blood was diluted in various proportions with deleucocyted blood, and two volumes of each of these dilutions were mixed with one volume of staphylococcus suspension and incubated for 15 minutes at 37° C. The amount of phagocytosis which had

taken place was then estimated in the usual manner with the following results:

Dilution of blood with deleucocyted blood.	Count per 100 leucocytes.
Undiluted	4·71
1 in 2	4·47
1 in 3	4·71
1 in 5	4·32
1 in 10	4·35
Deleucocyted blood only	No leucocytes.

It would appear, therefore, that a reduction in the number of leucocytes makes no appreciable difference to the number of microbes picked up by the individual leucocytes.

INFLUENCE OF THE NUMBER OF LEUCOCYTES IN BLOOD ON ITS BACTERICIDAL POWER.

As deleucocyted blood has been shown to have no bactericidal power to staphylococci, a method was at hand for the study of the relation of the number of leucocytes to the bactericidal power of blood. It has been shown above that so far as could be ascertained there was no alteration in the anti-bactericidal properties of the serum of deleucocyted blood, and in fact the only alteration was that the leucocytes had been removed.

Such a fluid, therefore, was very valuable as a diluent for blood, in that the chemical and physical characters of the blood remained unaltered, but the number of leucocytes progressively diminished as the dilution increased. The bactericidal tests were done in slide cells into which 50 c.mm. of blood or blood diluted with deleucocyted blood were placed along with 2·5 c.mm. of a suitable dilution of a staphylococcal broth culture. After incubation the number of colonies developing was enumerated, and the following result was obtained:

Dilution of blood in deleucocyted blood.	Colonies.	Percentage survivors.
Undiluted	9	5
1 in 2	22	12
1 in 3	30	17
1 in 5	64	37
1 in 9	98	56
Deleucocyted blood only	178	100

Implant per cell was 175 cocci.

These figures show that the bactericidal power of blood to staphylococci varies directly with the number of leucocytes, and that the killing power of normal blood is proportionate to the number of leucocytes present.*

* This subject of the relation of the number of leucocytes to the bactericidal power of the blood has been investigated by my colleague Dr. Maclean, and formed the subject of a communication to the Pathological Section of the Royal Society of Medicine in March last.

It has already been shown that the number of bacteria ingested by any individual leucocyte is independent of the number of leucocytes present, and if we assume, as is probably the case, that phagocytosis is the prime factor in the killing of bacteria such as staphylococci by blood in a slide-cell, then the result obtained as set forth above is exactly what we should expect on theoretical grounds, as the *total amount* of phagocytosis would vary with the number of leucocytes, and so the amount of killing of bacteria.

If, however, normal salt solution is used as a diluent for blood and the bactericidal power tested in the same way quite a different result is obtained. The bactericidal power of the blood diluted with salt solution does not fall off in proportion to the dilution, as is shown in the following experiment: Blood was diluted with normal salt solution, and of each dilution 50 c.mm. were mixed with 2·5 c.mm. of a suitable dilution of a staphylococcus culture and placed in slide-cells. The number of colonies developing was as follows:

Dilution of blood in normal salt solution.	Number of colonies which developed.
Undiluted	6
1 in 2	2
1 in 3	4
1 in 4	2
1 in 5	4
1 in 6	7

Implant of cocci per cell = 185.

Of course when blood is diluted with salt solution the physical conditions under which the leucocytes are acting are very considerably changed. The serum is diluted, but such a small dilution would make little difference to its opsonic power when so few microbes are introduced. The number of red corpuscles is diminished according to the dilution, and it is probable that this affects the result. It has been shown (Fleming, 1908) that in an opsonic mixture dilution of the washed corpuscles with normal salt solution induces a considerable increase in the amount of phagocytosis. It has also been shown that agglutination of the red corpuscles, which probably has a similar effect in clearing these out of the way, has a like effect. It does not seem at all unlikely that with fewer red corpuscles in the way the leucocytes and bacteria would come more easily into contact, with resultant phagocytosis and destruction of the bacteria.

The above results indicate clearly that when it is desired to investigate leucocytic function, deleucocyted blood furnishes a very much better diluent fluid than does normal salt solution.

All the above experiments refer to defibrinated blood. If citrated blood is used the leucocytes are filtered out in exactly the same way, and at the same time practically the whole of the platelets are removed. Fresh blood can be filtered before it clots and the leucocytes and platelets are retained in the filter. Such filtered blood clots only slowly. In filtering unclotted blood the filter frequently becomes clogged, so that great pressure is necessary to drive

the last portions of the blood through, resulting in considerable breaking up of the red corpuscles.

In addition to furnishing a convenient diluent for blood in experiments on leucocytic function, deleucocyted blood might be advantageously used in other ways, such as in the production of a pure hæmolytic antiserum, or as a culture medium for such microbes as would grow well in blood except for the fact that they are picked up and destroyed by the leucocytes.

SUMMARY.

A simple method is described for the removal of leucocytes (and platelets) from blood without altering the numbers of red corpuscles or removing the antibodies from the serum.

This deleucocyted blood has no bactericidal power to microbes such as staphylococci which are not killed by the blood fluids. It makes an admirable diluting fluid for blood when it is desired to vary the numbers of leucocytes, and for this purpose is much superior to normal salt solution in that the physical conditions are unaltered.

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FURTHER STUDIES ON STREPTOCOCCI FROM SCARLATINA, ERYSIPELAS AND PUERPERAL FEVER.

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Received for publication July 17th, 1926.

FOR some years evidence has been accumulating to show a specific grouping in strains of hæmolytic streptococci from cases of scarlet fever. More recently evidence has appeared pointing to similar grouping in strains from erysipelas and puerperal fever. Since the introduction of the Dick test, which has as its basis the production of a toxic substance in cultures of the scarlet fever streptococcus, it has been claimed that similar properties may attach to streptococci other than those for scarlet fever.

It seemed advisable, therefore, to inquire how general in the group of hæmolytic streptococci is the ability to produce a toxic filtrate, and what

relationship, if any, this bears to specific agglutination and the neutralizing effect of scarlet fever antitoxic serum on the various toxic filtrates.

For the purpose of this work seventy-four strains were collected, comprising eighteen strains from scarlet fever, sixteen from erysipelas, twenty-nine from puerperal fever and eleven miscellaneous strains.

With these strains agglutination tests were made, filtrates prepared and tested and neutralization experiments carried out.

TECHNIQUE.

Toxic Filtrates.

These were prepared by growing the strain for four days in Douglas tryptic digest broth enriched with a small quantity of sheep's blood. This medium was found to possess advantages in strength of toxic filtrate produced over other media investigated. The cultures were passed through a Berkefeld filter, and the resulting filtrates tested, after ascertaining their sterility, on known Dick positive reactors, 0·1 c.c. of a 1 in 1000 dilution in saline being injected intracutaneously in the forearm. A 1 in 1000 dilution was adopted as a standard for all filtrates since it was found that all scarlet fever strains produced a demonstrable toxin when so diluted, only one strain (Mac V) failing to react in greater dilution. Filtrates that gave a negative skin-reaction in a 1 in 1000 dilution were tested in dilutions of 1 in 100 and 1 in 10, control tests being made in each case with boiled filtrate in 1 in 1000 dilution.

Readings of tests were made between 12 and 24 hours after inoculation.

Sera for Neutralization of Toxin.

Anti-scarlatinal horse-serum (refined globulins) produced for clinical purposes was used for the most part.

Antitoxic sera were also produced in rabbits by injecting live organisms in 5 c.c. melted agar subcutaneously. For the first dose 0·1 c.c. of living broth culture was used, the amount increasing with each succeeding injection. The size of dose that could be tolerated varied considerably with individual animals. When a test-bleeding was found to yield a serum which would neutralize its homologous toxin the animal was bled and the serum stored in the cold. Sera were produced for scarlet fever, erysipelas and puerperal fever. The time required to produce an immune rabbit serum varied from ten to twelve weeks.

Sera for Agglutination.

These were produced in rabbits by saline suspensions washed from cultures grown on serum agar slants in increasing doses given on three successive days followed by a resting interval of three days. For the first three doses suspensions heated at 56° C. for one hour were given; for all the others living

TABLE I.—*Showing Agglutination Results by Groups, with Ability of Strains to Produce Toxic Filtrates and their Neutralization by Scarlet Fever Antitoxin.*

Strains.	Toxin production.	Neutralization of toxin by scarlet fever antitoxin.	Agglutination by immune rabbit sera.							
			Scarlet fever.		Erysipelas.		Puerperal fever.			Miscellaneous.
			Bashford.	Dochez.	New York.	Eg.	Collison.	Saxton.	Skinner.	Strathy.
Scarlet fever:										
Mac U—	+	+	640	640	80	80	40	40	80	40
G	+	+	640	320	160	80	80	40	80	—
E_{10}	+	+	320	640	40	40	20	20	40	20
R_5	+	+	320	320	80	40	40	20	20	20
L	+	+	640	640	20	20	80	40	20	—
S	+	+	1280	320	160	80	160	160	80	—
O_2	+	+	320	320	20	40	20	20	20	20
Turnbull	+	+	640	640	160	80	20	20	20	40
Moon	+	+	640	320	320	320	—	—	—	—
Bashford	+	+	2560	640	40	40	—	—	—	20
McGurn	+	+	320	320	20	20	—	20	20	40
Jeanurette	+	+	160	160	20	20	160	160	80	80
Cousineau	+	+	640	160	—	—	20	20	40	80
Baby L—	+	+	160	640	—	—	—	—	—	—
1989	+	+	320	160	20	20	—	—	—	—
Gehrke	+	+	640	320	40	40	—	20	—	20
Phair	+	+	1280	320	80	80	40	—	20	—
Dochez	+	+	1280	2560	160	160	40	20	—	—
Erysipelas:										
Roach	±	+	40	20	1280	1280	40	40	40	20
Flaherty	—	...	40	40	1280	640	80	40	40	40
New York	+	+	80	40	2560	2560	160	160	80	40
Davis	—	...	—	—	640	640	20	20	20	20
Wm. Smith	+	+	—	20	320	160	80	80	40	—
H. S. C—	+	+	40	40	320	320	40	40	40	—
T. G. H—, No. 1	±	+	80	80	640	320	20	20	—	20
T. G. H—, No. 2	—	...	80	80	640	640	20	20	—	20
1634	±	+	—	20	320	320	40	40	—	—
1774	±	+	—	—	—	—	—	—	—	—
1801	+	+	20	40	320	640	20	40	20	40
E_1	±	+	160	160	1280	640	80	40	80	20
E_2	±	+	80	80	1280	1280	40	40	20	40
E_3	+	+	160	80	640	2560	20	20	80	40
E_4	±	+	320	320	1280	1280	40	40	40	20
E_5	+	+	40	40	1280	1280	40	40	20	20
Puerperal fever:										
Mould	+	+	80	40	80	80	320	320	320	20

E_4	±	+	320	320	1280	1280	40	40	20	20	
E_5	+	+	40	40	1280	1280	40	40	20	20	
Puerperal fever:											
Mould	+	+	80	40	80	80	320	320	320	80	
Close	+	+	20	20	160	160	160	160	160	40	
Saxton	±	+	40	40	80	80	320	2560	320	80	
Buckingham	±	+	160	160	160	160	1280	1280	1280	160	
Holoway	+	+	40	40	40	40	160	160	80	20	
Collison	+	+	20	20	40	20	2560	80	80	40	
London	+	+	—	20	20	20	80	80	80	—	
Burns	+	+	—	—	20	—	160	160	160	—	
Hackman	—	. .	40	40	80	80	320	160	160	80	
Austin	+	+	160	160	160	160	320	320	320	160	
Everidge	±	+	40	40	80	80	320	320	320	320	
Harris	+	+	20	20	40	40	1280	1280	1280	—	
Halsey	+	+	—	—	—	20	80	160	80	—	
Speechley	—	...	20	20	40	40	320	640	640	80	
Bendon	—	...	—	—	20	20	320	320	320	20	
Hynson	—	...	—	—	—	20	320	160	160	20	
Rosenfield	+	+	—	40	40	40	640	640	640	—	
Crudgington	+	+	20	40	80	80	640	320	640	—	
Craddock	—	...	80	80	160	160	320	320	640	—	
Everidge	±	+	40	40	20	20	640	640	640	—	
Franklin	+	+	80	80	40	40	1280	1280	1280	20	
Wicks	±	+	160	160	80	80	640	640	640	20	
Edmunds	—	...	—	—	—	20	80	80	80	—	
Moore	—	...	20	40	40	40	320	320	320	—	
Turnham	—	...	40	80	40	40	640	640	640	40	
Foster	±	+	20	20	20	20	320	320	320	40	
Saxton	+	+	40	20	40	40	1280	1280	1280	20	
Skinner	—	...	—	20	20	20	320	640	2560	—	
Sampson	—	...	—	—	40	40	640	640	640	—	
Miscellaneous:											
Strathy	+	+	80	80	40	20	40	80	—	2560	
Smith	+	+	40	80	160	80	20	40	—	80	
963	—	...	—	—	80	20	20	80	40	40	
McC—	—	...	20	—	80	—	20	20	20	—	
McK—	—	...	40	80	40	20	40	40	80	20	
Brooks	—	...	80	80	160	160	80	80	160	—	
Ensoll	—	...	160	40	20	20	40	40	40	160	
Wallace	—	...	—	20	20	20	20	40	20	40	
Lud R—	+	+	80	—	—	—	—	—	—	20	
B. C. R—	—	...	—	80	80	80	80	40	80	160	
Inquest	+	+	40	20	40	40	40	20	20	40	

Under toxin production, + = Positive in dilution of 1 in 1000 or over.
± = " " 1 in 10, or 1 in 100.
— = Negative in dilution of 1 in 10.

Under agglutination—Figures represent titre in which agglutination was complete.
— = No agglutination.

organisms were used. When an animal was found to have produced a serum with a titre of 1 in 2560 it was bled and the serum stored in the cold. Before starting inoculation all animals were tested for the presence of non-specific agglutinins.

Eight immune sera were prepared—two from scarlet fever, two from erysipelas, three from puerperal fever and one from a case of acute pericarditis.

Neutralization of Toxin.

Equal parts of toxin dilution and serum dilution were mixed and allowed to remain in the incubator for two hours with frequent shaking. At the end of this time 0·1 c.c. of the mixture was injected intracutaneously. The dose 0·1 c.c. contained two skin-test doses of toxic filtrate.

Sera were diluted 1 in 10 in saline before mixing with toxin dilution.

Owing to the difficulty in securing volunteers for test purposes it was found necessary to use myself for the most part for testing of toxic filtrates and their neutralization.

Neutralization experiments were read in 24 hours after inoculation and again in 48 hours.

Neutralization was read as "complete" when there was absence of an area of redness at the point of inoculation at 24–48 hours.

Technique of Agglutination.

Suspensions for agglutination were prepared by growing the organisms on serum agar slopes overnight and removing the growth in a small quantity of distilled water. Clumps were broken up by forcing the suspension through a capillary pipette against the bottom of the tube. They were then suspended in suitable density in distilled water.

Serum dilutions were made in 0·4 per cent. saline ranging from 1 in 10 to 1 in 1280. Equal parts (0·5 c.c.) of suspension and serum dilution were used in the tests, making the final serum dilutions range from 1 in 20 to 1 in 2560 with a saline concentration of 0·2 per cent. A normal rabbit-serum control was set up with each test serum.

A control tube of equal parts of saline and bacterial suspension was also set up for each strain. Each serum was tested with its homologous strain and every other strain in the series.

In cross-agglutination experiments the homologous strain was included with the others for purposes of comparison.

Readings were made at the end of three hours in the water-bath at 56° C. It was not found satisfactory to use longer exposure in the water-bath or to allow the tubes to remain standing for any considerable time after removing them, since in each case a tendency to flocculation in the control tubes appeared which made interpretation of results difficult and unsatisfactory.

A number of freshly-isolated strains showed so persistent a tendency to agglutinate spontaneously in any of the suspending media investigated that they were discarded and do not appear in this report.

The results of these experiments appear in Table I.

DISCUSSION.

A careful study of this table reveals several points of interest. All the strains from scarlet fever produced toxic filtrates giving a positive skin-test in dilutions of 1 in 1000 or over. Six of sixteen strains from erysipelas, thirteen of twenty-nine strains from puerperal fever and four of eleven miscellaneous strains have also produced toxic filtrates that seem to differ from those from scarlet fever only in that they were weaker, as judged by skin reactions. When dilutions of 1 in 10 were used a larger number of non-scarlatinal strains were found to produce toxic filtrates.

Owing to the difficulty encountered in securing volunteers for skin-tests, it is impossible, within the scope of this work, to decide definitely whether there is any difference in the type of skin reaction among individuals when the toxic filtrates from various sources are used. Whenever available, however, individuals other than myself have been utilized and there would seem to be no difference.

Several workers have obtained toxic filtrates from strains from sources other than scarlet fever. Williams (1925) found that a strain from bronchitis, another from osteomyelitis and 42·8 per cent. of strains isolated from a series of excised tonsils yielded toxic filtrates that were neutralizable by convalescent scarlet fever serum and by immune horse-serum. All the strains from scarlet fever in her study also produced toxic filtrates that were neutralizable. Other strains in her series were found either not to produce toxic filtrates or unneutralizable ones. Birkhaug (1925), working with strains from erysipelas, found that a toxic filtrate giving a skin reaction similar to scarlet fever toxin could be obtained from a large number of his strains. These filtrates were not neutralized by scarlet fever antiserum, but were so by convalescent erysipelas serum or erysipelas anti-streptococcic rabbit or donkey serum. Lash and Kaplan (1925) have obtained toxic filtrates from puerperal strains.

It would seem, then, that the ability to produce a toxic filtrate capable of giving a skin reaction not distinguishable from the Dick test is not confined to strains from scarlet fever, but is rather widely distributed through the entire group of hæmolytic streptococci. There are at least two possibilities: first that all strains producing a toxic filtrate neutralizable by scarlet fever antiserum are essentially scarlet fever strains regardless of the pathological condition in which they are found; and that scarlet fever is but one of many conditions capable of arising from an infection by *Streptococcus scarlatinæ*. This would, of necessity, assume an immunity to scarlet fever in persons so infected. Birkhaug's results, however, point to the toxins from erysipelas and scarlet fever being separate entities. A second explanation points to the possibility that so far as toxin production by hæmolytic streptococci is concerned, the property is a generic one. No strict parallel can be found. But in this regard the findings of Hall and Stone (1916), working with the diphtheroid bacillus of Preisz-Nocard, are interesting, and lend some weight to the theory. They found that the bacillus was capable of producing a soluble toxin which was fatal for guinea-pigs, and that the toxic action was, to some extent at least,

neutralizable by diphtheria antitoxin. This is further borne out by the fact that in the present work scarlet fever antiserum was found to neutralize all toxic filtrates obtained.

When, however, neutralization experiments were made using immune sera produced in rabbits for toxin producing strains from scarlet fever, erysipelas and puerperal fever, there seemed to be a degree of specificity shown in that these sera neutralized their homologous toxins but exhibited very little cross neutralization. Birkhaug has pointed out that toxic filtrates from erysipelas are not neutralized by scarlet fever antiserum but are neutralized by erysipelas rabbit or donkey serum. The reason for this is not very clear. But it seems probable that an explanation may be found in the fact that in producing high-titre anti-scarlatinal horse-serum a lengthy course of immunization is necessary. In this connection it has been suggested that a greater number of group antibodies develop than when a shorter course of immunization is given; but actual proof of this is not forthcoming. It is taken as accepted, on the other hand, that the production of certain antisera, *e.g.* agglutinating sera, the horse is not a suitable animal owing to the fact that group agglutinins are often present previous to immunization.

It is quite generally agreed by workers who have studied the specific agglutination of *Streptococcus scarlatinæ* that this method serves to differentiate these organisms from other hæmolytic streptococci. This is also true when strains from other pathological sources are considered. Birkhaug (1925) showed that 91·2 per cent of strains studied from erysipelas fell into a group distinguishable from strains from scarlet fever, puerperal fever, tonsillitis and a variety of other sources. Stevens and Dochez (1926) also found that erysipelas strains formed a closely related group, equally as compact as scarlatinal strains, though related to them antigenically. In an earlier communication (1924) it was shown that strains from scarlet fever and erysipelas form distinct groups, and that puerperal strains also form a separate group by agglutination. The present work confirms these earlier observations of my own and of those who have made similar investigations, and adds additional evidence to the specificity of the puerperal grouping. Sufficient experiments have not been made by absorption methods to determine whether sub-groups exist within these groups. It is difficult to explain why puerperal strains should form a separate group, since puerperal fever is not considered as a disease entity in the same way as scarlet fever and erysipelas, unless one considers the effect that environment may have on their agglutination reactions. Douglas, Fleming and Colebrook (1920), in a study of hæmolytic streptococci occurring in war wounds, found they constituted a distinct group by agglutination. It may well be, then, that the puerperal state on the one hand and the peculiar tissue conditions in war wounds on the other offer an explanation of this specific grouping.

Evidence has been brought forth recently to show that toxin production and agglutination are to some extent closely related. Stevens and Dochez (1926), in a study of strains from acute pharyngitis among nurses, have found that strains which produced toxic filtrates and were neutralized by scarlet fever antiserum were proved to be scarlatinal strains by agglutination methods, and concluded that toxic production and agglutination were closely parallel. But

this does not seem to be the case when a large number of strains from a variety of sources are investigated as shown in the present study.

CONCLUSIONS.

All strains of hæmolytic streptococci from scarlet fever produce toxic filtrates as judged by intracutaneous tests. A large number of strains from erysipelas, puerperal fever and from various other pathological conditions produce a similar toxic filtrate.

All toxic filtrates, regardless of their source, are neutralized by scarlet fever horse-serum. Results obtained when immune rabbit sera from various toxin-producing strains are used for neutralization may indicate, however, higher specificity.

By agglutination three main groups are shown to exist among hæmolytic streptococci. The scarlet fever and erysipelas groups are confirmed and a similar grouping shown among strains from puerperal fever.

Toxin production and agglutination by scarlet fever immune sera are not closely related when strains from a variety of pathological sources are studied.

The author wishes to thank the following:

Dr. J. C. G. Ledingham for much kindly help and criticism during the course of this work.

The Bacteriological Department of St. Mary's Hospital for strains from puerperal fever.

Dr. Franklin Stevens, New York City, for several strains from erysipelas.

The Connaught Research Laboratories, Toronto, Canada, for scarlet fever antitoxin used in neutralization experiments.

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OBSERVATIONS ON SIX ANOMALOUS BLOODS, WITH REFERENCE TO THE THEORY OF ISOAGGLUTINATION.

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Received for publication July 23rd, 1926.

THE present communication embodies the results of the examinations of 120 bloods in reference to their conformance to the hypothesis of Landsteiner (1901) as to the constitution of the blood groups. This author gave good ground for the belief that all human bloods could be divided into groups according as to whether his hypothetical agglutinable bodies, which may be referred to as *A* and *B*, occurred together, singly, or not at all in the corpuscles, and the corresponding agglutinins *a* and *b* in the sera. This hypothesis has gained general credence, in spite of the occurrence of apparent anomalies, and still forms the basis of the technique of the grouping of blood for the purpose of transfusion.

The investigation was undertaken with the view of discovering bloods giving anomalous reactions, and, if possible, of determining their constitution.

Three means were used for the identification of anomalous bloods. In the first place all available sera were tested against all available red cells. As more than six specimens of red cells were seldom available at one time the application of this procedure was obviously limited. In the second place a series of cross-agglutinations were performed. The bloods were tested in this way in groups of twelve, all the sera of each set being put up for agglutination with all the corpuscles, irrespective of group. In all, four sets of cross-agglutinations were performed, involving the examination in this way of forty-eight bloods. The third method, that of absorption of sera by corpuscles, proved the most successful in disclosing anomalous bloods. Many single sera of all groups were examined in this way as the occasion offered, and in addition twelve sera of Group IV were absorbed, each by six specimens of cells of Groups II and III. The absorbed sera were then tested for their agglutinating power on all the corpuscles used in the absorption.

All the agglutinations, both with absorbed and unabsorbed sera, were performed by the microscopic method described by the author in a previous paper (1922). In absorbing sera the cells were used in a 50 per cent. emulsion saline. This emulsion was mixed with an equal quantity of serum; the mixture was well shaken, allowed to stand at room temperature overnight, and again shaken in the morning; the red cells were thereafter removed by centrifugalization.

In the course of the investigation six anomalous bloods were encountered. It is proposed to describe the reactions given by these in so far as they were investigated, and to draw certain inferences as to the constitution of the blood groups from their behaviour.

Blood No. 6.—Red cells were agglutinated by ten Group III sera. They failed to agglutinate with the two sera of Group II (1*a* and 40), but were powerfully agglutinated by two other sera of Group II (4 and 7).

The serum of this blood failed to agglutinate all cells of Groups II or III with the exception of one specimen of Group III (14), with which it gave a trace of agglutination.

Absorption of a Group IV serum by the cells of this blood resulted in the complete removal of both agglutinins *a* and *b*, as shown by subsequent tests against cells of Groups II and III.

The red cells of this blood evidently contained both the agglutinable factors *A* and *B*, and so far as the cells were concerned it was therefore of Group I. The failure on the part of the Group II sera 1*a* and 40 to reveal the factor *B* may be accounted for by a low titre of the agglutinin *b* in these sera and a low concentration of the factor *B* in the cells. That the sera 1*a* and 40 were deficient in the agglutinin *b* was shown by titrating these sera against several samples of red cells of Group III. In no instance did agglutination take place in dilutions over 1 in 10 of the serum.

In spite of the Group I constitution of its red cells the serum of this blood contained agglutinin *b*, and in respect of its serum it belonged to Group II. The fact that auto-agglutination of the red cells did not occur in this blood was presumably due, as in the further cases to be described, to the relatively low titre of the serum and the small proportion of the corresponding agglutinable factor in the cells.

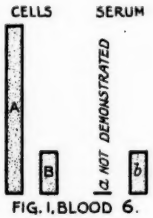

FIG. 1, BLOOD 6.

FIG. 2, BLOOD 15

FIG. 3, BLOOD 20.

FIG. 4, BLOOD 22.

FIG. 5, BLOOD 59.

FIG. 6, BLOOD 83

Graphically the construction of this blood may be expressed as in Fig. 1. In this case, as in all subsequent diagrams, the relations are entirely arbitrary.

Blood No. 15.—The corpuscles tested with seven sera of Group II showed no agglutination with five, but agglutinated powerfully with one (7) and slightly with a second (20). Agglutination occurred with all Group III sera used.

The serum agglutinated all samples of corpuscles of Group III, and failed to agglutinate all of Group II against which it was tested.

Absorption of a Group IV serum by cells of this blood resulted in complete removal of both agglutinins *a* and *b*, as shown by failure on the part of the absorbed serum to agglutinate cells of Groups II and III and also cells 20 and 22 (anomalous Group I).

The constitution of this blood would appear to be the same as that of Blood 6, the only difference being a higher titre of the agglutinin *b* in the serum (Fig. 2).

Blood No. 20.—The cells of this blood were agglutinated by two sera of Group III, but by none of Group II against which they were tested, four in all. The serum agglutinated cells of Group III but not of Group II. It therefore appeared to be a blood of Group II. That it was not a simple member of this group was shown by the behaviour of the cells when used to absorb a serum of Group IV. Absorption of such a serum resulted in complete removal of both agglutinins, as shown by subsequent test against cells of known Groups II and III.

Graphically the constitution of this blood would be as in Fig. 3.

Blood No. 22.—The cells of this blood were agglutinated by four sera of Group II, but failed to agglutinate with two others (1*a* and 17). They were strongly agglutinated by all Group III sera used.

The serum agglutinated all cells of Group III against which it was put up, but failed to agglutinate cells of Group II.

A serum of Group IV separately absorbed by two specimens of cells of Group II remained capable of agglutinating cells 22.

The constitution of this blood would be as in Fig. 4.

Blood No. 59.—The cells of this blood were agglutinated by all the Group II sera with which they were tested, but by none of those of Group III. The serum agglutinated cells of Group II but not those of Group III. On this evidence alone the blood appeared to belong to Group III.

The cells were used along with four specimens of cells of Group II and one of Group III in the absorption of five sera of Group IV. The behaviour of all the cells of Group II and those of Group III was as expected, but the cells of Blood 59 gave the following result:

Sera IV after Absorption by Cells 59.

Sera.			54	57	60	39	46
Against cells	II	55	±	±	±	±	—
		56	—	—	—	—	+
		58	+	—	—	+	—
		63	+	—	—	±	±
	III	59	—	—	—	—	—
		61	—	—	—	—	—

Cells of Blood 59 behave as though belonging to Group III in that they remove the agglutinin for Blood 61 as well as for themselves; they also,

however, to some extent remove the agglutinins for the Group II cells. The fact that in no case is removal complete may be accounted for by relative differences in the titre of the agglutinin *a* in the Group IV sera used and in the concentration of the factor A in the Group II cells. Unfortunately, sufficient material was not available for the performance of reabsorptions.

The constitution of Blood 59 is shown in Fig. 5.

Blood No. 83.—The behaviour of the cells and serum of this blood so far as agglutination was concerned was as though it were a member of Group III. Indication of the presence of agglutinable factor A became evident in the course of the absorptions of Group IV sera with the cells of this blood. With two Group IV sera (81 and 84) the cells behaved as would be expected of cells of Group III, that is to say the power of agglutinating cells of members of this group was lost, while the sera remained capable of agglutinating cells of Group II. When used to absorb a further serum of Group IV (80), however, the cells removed both agglutinins *a* and *b*, so that the serum lost not only its power of agglutinating cells of Group III but of Group II also. On testing serum 80 against several Group II cells it became apparent that the agglutinin *a* was present in very low titres. In the case of Blood 83 it would then appear that in addition to the readily recognizable agglutinable factor B, a small proportion of factor A was also present, sufficient to remove all the *a* agglutinin from serum 80, in which its titre was low, but not sufficient to remove it entirely from the other Group IV sera used.

This blood would be constituted as in Fig. 6.

DISCUSSION.

Of the six bloods described above, four on superficial examination appeared to belong to Group II and the remaining two to Group III. If the presence of both the agglutinable factors A and B in the cells be accepted as the criterion of a Group I blood, they all actually belonged to Group I.

Good reason was given by von Dungern and Hirschfeld (1910) for the belief that the factors A and B are Mendelian dominants, and much further evidence has been produced to this effect by later workers. So firmly does this now appear to be established that in several recent instances the appearance of these dominants in parents and offspring has been accepted as evidence in German courts of law in cases concerned with the establishment of paternity (Schiff; personal communication). It has been pointed out by the present author (1923) that the proportion of Group I individuals in the population is much smaller than should be the case, allowing for the free play of the dominants A and B in general matings. It was suggested that the explanation of this might lie in the fact that certain combinations of A and B in the gamete constitute a lethal factor. The true explanation would appear to be that this rarity of individuals of Group I in the general population is apparent and not real. As pointed out above, of the six individuals dealt with in this communication four would ordinarily have been accepted as belonging to Group II, while two would have been relegated to Group III. As a matter of fact, in so far as the red cells of all contained both factors A and B all belonged to Group I. If the assumptions

made above are correct it must be the fact that many individuals of Group I are included by the methods ordinarily in use to-day under Groups II and III.

It has been pointed out by the present author in a previous communication (1922) that the extent to which the agglutinable factors A and B are present in any corpuscles is capable of very great variation, and that the same holds for the agglutinin titre of the serum. Jones and Glynn (1926) have recently made extensive observations upon this point and have attempted to express the variations in numerical form. This fact would give ground for the belief that in the cells of certain bloods one or other of the agglutinable factors may be present to such a small extent as to escape detection altogether on superficial examination. This is merely to state in other words the author's contention that many bloods now accepted as belonging to Groups II or III are really of Group I.

The principle of the blood groups finds its chief practical application in the selection of bloods for transfusion. Of the six bloods described above the four apparently belonging to Group II would theoretically be capable of producing symptoms if administered to a Group II recipient, and the same holds for the two apparently belonging to Group III if given to a member of that group. Whether or not they would actually have produced symptoms depends entirely upon the height of the agglutinin titre in the serum of the recipient. Probably few individuals would be found with a serum having a sufficiently high agglutinin titre to produce any marked effect upon cells having so low a proportion of agglutinable factor as to permit of its being missed by the ordinary agglutination tests. None the less, this is a further indication of the necessity already pointed out by the present author (1922) of using sera of a high titre for the performance of agglutination tests in the selection of donors for transfusion. The facts detailed above would suggest that many of the reactions following upon the transfusion of apparently compatible blood are due to the presence of incompatible agglutinable factors which had escaped detection in the preliminary tests—in fact, to the administration of a blood of Group I. If the deductions drawn above are correct, the only entirely safe way of selecting a donor is by testing his red cells against the serum of the proposed recipient.

Some of the facts detailed above are capable of explanation in a manner other than that offered by the author. Guthrie and Huck (1923), faced by similar anomalies in bloods coming to light in the course of an exceedingly extensive and laborious series of observations, explained them by postulating a further agglutinable substance C with its corresponding agglutinin *c*. Simson (1926) has recently confirmed this observation. The attention of the writer had not been drawn to the work of Guthrie and Huck at the time that the above investigations were in progress and therefore no definite search was made for the new factors. Of the bloods described above the reactions of only two, Nos. 22 and 83, are explicable by the postulation of further agglutinable factors or agglutinins, and in the case of Blood 83 an explanation on these lines would be rather forced. For the behaviour of the remaining bloods the only explanation seems to be the possibility of the existence in the same blood of an agglutinable factor in the red cells and its corresponding agglutinin in

the serum, but both of a titre insufficient to bring about agglutination of the red cells.

The observations detailed above point to the conclusion that in the absence of absorption tests many bloods of Group I will escape detection. This fact is of importance (1) in that it offers an explanation of the apparent rarity of bloods of Group I; (2) in its bearing upon the matching of bloods for transfusion; and (3) in its medico-legal applications.

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INSULIN IN NORMAL AND CANCEROUS TISSUES.

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Received for publication July 30th, 1926.

IT has been shown by various workers that insulin is contained in tissues other than the pancreas. The distribution of this substance in various tissues in man and animal was investigated by Baker, Dickens and Dodds (1924), and it was found that good yields can be obtained from the kidney, spleen, salivary glands, muscle and liver. The main investigation was confined to normal tissues, and only one pathological case was examined. The patient in this instance died of diabetic coma before any treatment could be instituted, and on extracting the organs obtained shortly after death it was found that insulin could be recovered from all the tissues, including the pancreas.

In view of these interesting results it was felt that an extension of this work to other pathological tissues might also be of interest, and in this communication investigations on malignant tumours are described. While this

work was in progress a paper was published by Cori (1925), in which it was stated that this investigator, using an alcoholic method of extraction, had failed to obtain any evidence of the presence of insulin in four malignant tumours, of which three were human material, while the fourth was a lympho-sarcoma of the dog.

In the work about to be described the acetone picric acid process of Dodds and Dickens (1924) was employed. This can readily be converted into a micro-method, with the result that very small weights of tissues can be extracted satisfactorily. The details of the technique are described in the paper by Baker, Dickens and Dodds (1924), and its application to the study of the insulin content of small amounts of tissues from fish islets has also been reported (Swale Vincent, Dodds and Dickens, 1924 and 1925).

In the first place animal tumours were employed for the investigation, and in the table below will be found the results. Observations upon the insulin content from the tissues of normal rats are also included in the table:

Tissue.	Weight in gm.	Yield of hydrochloride in mg.	Rabbit unit.	Yield in rabbit units per gm.
Jensen rat sarcoma	19·1	11·1	Inactive	0
,, ,,	45·0	20·0	,,	0
,, ,,	47·2	32·0	,,	0
Carcinoma 9	7·9	5·5	,,	0
,,	22·0	10	,,	0
Mouse carcinoma (113)	15·0	84	,,	0
,, ,, strain 63	12·9	51	,,	0
,, ,, ,, 206	12·0	40	,,	0
,, ,, ,, 113	10·0	22	,,	0
Twort	18·5	26·5	,,	0
Normal rat:				
Pancreas	3·7	18·0	12	0·4
Spleen	5·8	7	7	0

It can readily be seen that no active material could be obtained from any of the malignant tumours examined, whereas normal yields resulted from extractions by the same process from non-malignant tissues of the animals in question.

A series of similar observations were made upon malignant tumours removed from human patients. The material was collected from the operating theatre in an iced container and was immediately extracted by the acetone picric acid process. In all, seven tumours were extracted, four being carcinoma mammæ, two sarcomata and one epithelioma. No active material was obtained.

As pointed out in the opening section, appreciable yields of insulin may be obtained from normal human tissues extracted in the same manner. It would appear therefore that malignant tumours differ from normal tissues in being devoid of insulin.

Since the meaning of the presence of insulin in tissues other than the pancreas is as yet not understood, it is obviously not possible at present to draw definite conclusions from the absence of insulin in malignant new-growths. It is, however, significant that the work of Warburg has disclosed a parallel difference between the carbohydrate metabolism of normal cells and of the cells of malignant new-growths. The former oxidize carbohydrate, the latter split it with the formation of lactic acid. This parallelism, if not merely an accident, suggests that the oxidation of glucose by the cells is dependent on the presence of insulin at the site of oxidation, and that in the absence of insulin the cells metabolize glucose in a different manner with the formation of lactic acid. If this interpretation is correct, one would expect to find that the formation of lactic acid by normal cells, *e. g.* muscle, should be diminished by insulin. Macleod (1926), in his recent book, quotes experiments by Dudley, Kuhn and Baur and Collezo, Haendel and Rubeiro, which agree in showing that the muscle of rabbits after an overdose of insulin forms only about one-half of the amount of lactic acid found in the muscle of normal starved rabbits.

CONCLUSIONS.

Insulin is present in normal tissues in appreciable amounts. It is absent from malignant new-growths. The cells of normal tissues oxidize glucose, the cells of malignant new-growths split glucose, as Warburg found, with the formation of lactic acid. This parallelism suggests that the oxidation of glucose by the cells is dependent on the presence of insulin at the site of oxidation.

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THE PHOSPHATE AND CALCIUM MECHANISMS IN SKELETAL MUSCLE AFTER PARATHYROIDECTOMY.

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Received for publication August 3rd, 1926.

WHEN muscle substance is allowed to stand at room temperature, or at higher temperatures, and especially if the reaction is alkaline, there is a setting free of lactic and phosphoric acids in equimolecular proportions from a parent substance named by Embden "lactacidogen." Chemically this body is probably fructose-diphosphate. The phosphate is usually present in the muscle in part as this ester and in part in the free form. The ratio of the free to the bound may be increased by warming minced muscle, and complete hydrolysis of the ester may be effected by a temperature of 40° maintained for 2 hours if the reaction is kept alkaline. On the other hand, the proportion of the bound to the free may be greatly increased by the action of fluoride anions, and this synthetic process will go on to a greater extent if the forerunners of the ester, glycogen and phosphate, are added in amounts over those existing in the muscle. Fresh dog's muscle may thus synthetize, with glycogen present, 14·6 mgm. out of 28·0 mgm. free phosphate present in 1 gm. muscle. Under normal and various experimental conditions the relative amounts of the bound and free phosphate are found to vary only within comparatively narrow limits, being kept more or less constant by two opposing forces, one synthetic, the other hydrolytic. In this respect lactacidogen differs from glycogen, which shows considerable variation in the amount present; the former substance is therefore to be regarded as an intermediate body, and the latter as the storage material.

During a muscular contraction, chloride anions pass into the muscle-fibre and facilitate the splitting of lactacidogen into two molecules of lactic acid and of inorganic phosphate. In the restitution phase of the contraction one molecule of lactic acid out of every four is completely oxidized; the rest recombines with free phosphate to form lactacidogen. As the surface of the muscle-fibre is impermeable to fluoride anions no such exchange of ions takes place, and the phosphate is largely retained in organic combination. In addition, as the fluoride checks the hydrolytic process and also oxidation, but enables synthesis of the precursors of the ester to continue, the full synthetic power of the muscle can be exercised. The synthetic power of fluoride on the phosphate ester has been worked out for normal, adrenalinized and insulinized muscle by Milroy (1925), and for fatigued muscle by Andrews (1925), Embden and Jost (1925).

Removal of the parathyroid glands results in a marked disturbance of the neuro-muscular mechanism, characterized by certain signs and symptoms which are dramatically relieved by the exhibition of active parathyroid extract as prepared by Collip (1925). The rapidity of the disappearance of the symptoms suggests ionic changes in the muscles. The following series of experiments

was carried out to determine if the tetany of parathyroidectomized animals is associated with a disturbance of the hexose-phosphoric acid ester or with changes in the calcium and phosphorus contents of blood and muscle.

TECHNIQUE.

In each case, therefore, there were determined in the blood the amounts of calcium (Tisdall's method as modified by Collip and Clarke), phosphorus (Brigg's method), glucose (Folin), and in some cases the relative plasma-cell volume and the viscosity time. In the muscles the amounts of calcium, lactic acid and of phosphates were estimated. The muscle on removal was placed in a thin glass vessel, sitting on ice. Pieces about 1 gm. weight were minced and placed in various solutions. The muscle placed in 4% HCl. in saturated NaCl solution gave the amount of inorganic phosphoric acid present in the muscle at the time of killing. This has been called by Embden the *A* value. That present after heating the muscle in a weak bicarbonate solution for 2 hours at 40°C. gave the total inorganic phosphate—the *B* value. The synthetic action of the fluoride anion was obtained by letting the muscle stand at room temperature for three hours in the following solutions:

1. N/10 Na F + 2% sod. bicarbonate . . . . . *C* value.
2. N/10 Na F + 2% sod. bicarbonate + glycogen. . . . . *D* value.
3. N/10 Na F + 2% sod. bicarbonate + 5 mgm. H_3PO_4 (as Na_2HPO_4). *E* value.
4. N/10 Na F + 2% sod. bicarbonate + 5 mgm. H_3PO_4 + glycogen . *F* value.

In each case these solutions were freed from the muscle proteins in the following way: 10 c.c. of saturated sodium chloride containing 4% HCl. were added to each, and one hour later 20 c.c. of 5% mercuric chloride. After standing overnight the muscle was disintegrated in the mortar. The acid extract was filtered off under pressure, and the residue washed to remove all the acid-soluble phosphate. Hydrogen sulphide was then bubbled through to precipitate the mercuric chloride and the sulphuretted hydrogen removed by aeration. The filtrate was used for the estimation of phosphates by Embden's gravimetric method, and for the lactic acid according to Hirsch-Kaufman's directions.

The different values obtained were:

1. Pre-existing phosphate, (and in some) glycogen and lactic acid—*A* values.
2. Total phosphate—*B* value; and from this the bound phosphate (*B*–*A*), and maximal lactic acid.
3. Amount of pre-existing phosphate synthetized by fluoride (*A*–*C*), by fluoride and added glycogen (*A*–*D*), by fluoride and added phosphate (*A*–*E*), and by fluoride and added phosphate and glycogen (*A*–*F*).

ANALYSIS OF SIMPLE PARATHYROIDECTOMY.

Experiment 1.—A terrier was thyroparathyroidectomized. 72 hours later definite rigidity of the muscles developed, and the animal showed a reluctance to move about. The animal was killed on the fourth day. During the first stage of anæsthesia well-marked clonic movements were superimposed on the constant extensor spasm.

Analysis.

Blood:

Sugar	0·12%
Phosphorus	2·1 mgm. per 100 c.c.
Calcium	7·0 „ „ „

Muscle:

A	0·171% H_3PO_4
B	0·234% „
C	0·166% „
D	0·149% „

In *E* 5·7 mgm. H_3PO_4 were added to 1·271 gm. muscle, and 5·74 mgm. H_3PO_4 were recovered.

In *F* 5·7 mgm. H_3PO_4 were added to 1·861 gm. muscle, and 5·76 mgm. H_3PO_4 were recovered.

The ratio of the bound to the free phosphate is 27·0 : 73·0
After Na F the ratio is . . . . . 29·1 : 70·9

	Synthesis of phosphate expressed as percentage of original free H_3PO_4.	Actual amount synthetized per 100 gm. muscle.
Na F	2·9%	0·005 gm. H_3PO_4
Na F and glycogen	12·2%	0·022 „ „
Na F and H_3PO_4	27·0%	0·168 „ „
Na F and H_3PO_4 and glycogen	35·2%	0·168 „ „

Muscle calcium 6·0 mgm. per 100 gm. muscle.

The phosphate values, both free and combined, in parathyroidectomized muscle are lowered considerably below normal. The synthesis by fluoride is practically *nil*, but is rather better on the addition of phosphates. The calcium value is less than half the normal. These low muscle contents of phosphate and calcium are associated with a lesser amount of both in the blood.

Experiment 2.—Parathyroidectomy followed by the administration of active extract of the parathyroid gland.

Within 72 hours well-marked tetany indicated by tremors of the limbs, laryngismus and diarrhœa developed in a young dog following removal of the parathyroid glands.

The injection of an extract prepared according to Collip's directions caused the disappearance of the symptoms in a few hours. At first the animal was incapable of walking, but in this short time after administration of the extract it was running about apparently perfectly normal. Injections were continued for a period of 10 days. The symptoms returned if the extract was withheld more than 24 hours, but then promptly disappeared after its administration. Tremors were finally allowed to develop, and the animal was anæsthetized and bled. During the first stage of anæsthesia, clonic spasms of considerable severity were manifested on top of the original tetany.

Blood: *Analysis.*

Hæmatocrite	Plasma	58·1
	Cells	41·9
Phosphorus		5·0 mgm. per 100 c.c.
Calcium		5·2 „ „ „
Lactic acid		85·0 „ „ „
Sugar		0·14%

Muscle:

Phosphates.

A	0·188%
B	0·263%
C	0·137%
D	0·122%

In *E* 5·7 mgm. H_3PO_4 were added to 1·527 gm. muscle (*A* value 2·87 mgm.) and 6·22 mgm. H_3PO_4 were recovered.

In *F* 5·7 mgm. H_3PO_4 were added to 1·216 gm. muscle (*A* value 2·28 mgm.) and 7·98 mgm. H_3PO_4 were recovered.

The ratio of the bound to the free phosphate is 28·9 : 71·1

After Na F the ratio is 47·9 : 52·1

	Synthesis expressed as percentage of original free H_3PO_4.	Actual weight of H_3PO_4 synthetized per 100 gm. of muscle.
Na F	27·1%	0·051 gm. H_3PO_4
Na F and glycogen	35·1%	0·066 „ „
Na F and H_3PO_4	27·5%	0·154 „ „
Na F and H_3PO_4 and glycogen	22·0%	0·144 „ „

	Glycogen.	Lactic acid.
A	0·21%	0·13%
B	—	0·158%
C	0·034%	0·081%

Calcium 5·6 mgm. per 100 gm. muscle.

The quantity of phosphate present in the muscle is small, and synthesis is poor, but slightly better than in No. 1. The muscle calcium is low, corresponding to a reduced amount of calcium in the blood.

EFFECT OF PARATHYROID EXTRACT.

Experiment 3.—Thyroparathyroidectomy was followed by stiffness of muscles and laryngismus. Parathyroid extract was given twice a day for 3 days.

Analysis.

Blood:

Hæmatocrite	Plasma	48·8
	Cells	51·2
Viscosity time (serum)		3 mins. 10 secs.
Phosphorus		4·5 mgm. per 100 c.c.
Calcium		6·1 „ „ „
Sugar		0·33%

Muscle :

Phosphates.

A	0·211%
B	0·315%
C	0·188%
D	0·113%

In *E* 5·1 mgm. H_3PO_4 were added to 2·382 gm. muscle (*A* value 5·02 mgm.) and 5·4 mgm. H_3PO_4 were recovered.

In *F* 5·1 mgm. H_3PO_4 were added to 2·237 gm. muscle (*A* value 4·72 mgm.) and 6·81 mgm. H_3PO_4 were recovered.

Ratio of bound to free phosphate is	33·0 : 67·0
After Na F the ratio is	40·3 : 59·7

	Synthesis expressed as percentage of original free H_3PO_4.	Actual amount H_3PO_4 synthetized per 100 gm. muscle.
Na F	10·8%	0·023 gm. H_3PO_4
Na F and glycogen	46·4%	0·098 „ „
Na F and H_3PO_4	46·5%	0·198 „ „
Na F and H_3PO_4 and glycogen	30·6%	0·136 „ „

	Muscle glycogen.	Lactic acid.
A	0·626%	0·163%
B	—	0·370%
C	0·011%	0·107%

Muscle calcium 11·2 mgm. per 100 gm.

The exhibition of active extract tends to bring the phosphate and calcium mechanisms in blood and muscle back towards normal. Thus the quantity of phosphate is increased over that in Experiments 1 and 2, and synthesis, under the action of fluoride combined with glycogen and phosphate, is improved. The muscle calcium is about normal.

PARATHYROIDECTOMY RELIEVED BY INTRAVENOUS INJECTION OF CALCIUM CHLORIDE.

Experiment 4.—Thyroparathyroidectomy was followed by tetany in 24 hours. 10 c.c. of $CaCl_2$ were injected intravenously. This was repeated 12 hours later shortly before examination.

Analysis.

Blood :

Hæmatocrite $\frac{\text{Plasma}}{\text{Cells}}$	60 / 40	Slightly hæmolyzed.
Viscosity time	5·0 mins.	
Phosphorus	8 mgm.	per 100 c.c.
Calcium	12·2 „	„ „
Sugar	0·08%	

Muscle :
Phosphates.

A	0·204% H_3PO_4
B	0·381% ,,
C	0·177% ,,
D	0·165% ,,

In *E* 5·1 mgm. H_3PO_4 were added to 2·307 gm. muscle (*A* value 4·7 mgm.) and 6·869 mgm. H_3PO_4 were recovered.

In *F* 5·1 mgm. H_3PO_4 were added to 2·314 gm. muscle (*A* value 4·72 mgm.) and 7·27 mgm. were recovered.

Ratio of bound to free phosphate is . . . 46·5 : 53·5
After Na F the ratio is . . . . . 53·6 : 46·4

	Synthesis expressed as percentage of original free H_3PO_4.	Actual amount of H_3PO_4 synthetized per 100 gm. of muscle.
Na F	13·2%	0·027 gm. H_3PO_4
Na F and glycogen	19·1%	0·039 ,, ,,
Na F and H_3PO_4	62·3%	0·127 ,, ,,
Na F and H_3PO_4 and glycogen	54·2%	0·110 ,, ,,

Muscle calcium 13·0 mgm. per 100 gm.

The quantity of phosphate present is about normal, but the synthetic action is not good. The blood phosphorus is increased. The calcium content is normal, and is associated with a blood calcium slightly above the average.

PARATHYROIDECTOMY FOLLOWED BY THE INTRAVENOUS ADMINISTRATION OF CALCIUM CHLORIDE AND SODIUM PHOSPHATE.

Experiment 5.—Tetania parathyropriva developed in 72 hours. 10 c.c. of 3% $CaCl_2$ were injected along with 10 c.c. of 3% sodium phosphate (pH 7·4). This was repeated 12 hours later about one hour before examination.

Analysis.

Blood :

Phosphorus	6·3 mgm. per 100 c.c.
Calcium	12·0 ,, ,, ,,
Sugar	0·09%

Muscle :
Phosphates.

A	0·188%
B	0·311%
C	0·128%
D	—

In *E* 5·1 mgm. H_3PO_4 were added to 1·365 gm. muscle (*A* value 2·57 mgm.) and 5·50 mgm. H_3P_4O were recovered.

Lactic acid :

A	0·073%
B	0·220%
C	0·065%
Ratio of bound to free phosphate is	39·5 : 60·5
After Na F the ratio is	58·9 : 41·1

	Synthesis expressed as percentage of original free H_3PO_4.	Actual amount of H_3PO_4 synthetized per 100 gm. of muscle.
Na F	13·2%	0·060 grm. H_3PO_4
Na F and H_3PO_4	28·3%	0·159 „ „

Muscle calcium 20 mgm. per 100 gm.

The phosphate quantities are about the average, but synthesis is as in the other cases of parathyroidectomy. The lactic acid values vary with the phosphate values. The calcium value of the blood is slightly raised, and that of muscle is considerably above normal.

COMMENT.

The following table gives a summary of the *A*, *B*, *C* and *D* values and percentage synthesis of phosphate in normal and parathyroidectomized muscles.

	A value.	B value.	C value.	D value.	Synthesis per 100 gm. muscle.			
					Na F.	Na F and glycogen.	Na F and H_3PO_4.	Na F and H_3PO_4 and glycogen.
Normal 1	·280	·382	·134	·130	·146	·150	—	—
„ 2	·233	·390	·128	·143	·105	·090	—	—
„ 3	·259	·457	·067	—	·192	—	—	—
Parathyroidectomy 1	·171	·234	·166	·149	·005	·022	·168	·168
Parathyroidectomy 2	·188	·263	·137	·122	·051	·066	·154	·144
Parathyroidectomy and extract	·211	·315	·188	·113	·023	·098	·198	·137
Parathyroidectomy and $CaCl_2$	·204	·381	·177	·165	·027	·039	·127	·110
Parathyroidectomy and $CaCl_2$ and Na_2HPO_4	·188	·311	·128	—	·060	—	·159	—

The muscle of a parathyroidectomized animal shows a lessened amount of phosphorus, both in the free and combined forms. The synthesis under the action of fluoride is poor, but approaches that of normal muscle on the addition of more sodium phosphate, *e. g.* the normal muscle shows a synthesis under fluoride of 0·147% (average) against 0·033% (average) of the parathyroidectomized muscle.

These results can hardly be taken as causative in the production of tetany. They are more likely to be secondary to the fatigue induced in the muscle by the excessive and prolonged movements, as the values correspond fairly closely to those obtained after fatigue has been produced by stimulation of the sciatic nerve (Andrews, 1925).

The following table shows the relationship between the calcium and phosphorus contents of blood and muscle.

Blood.		Muscle.		
Phosphorus per 100 c.c.	Calcium per 100 c.c.	Phosphorus as H_3PO_4.	Calcium per 100 gm.	Remarks.
2·1 mgm.	7·0 mgm.	·234%	6·0 mgm.	Tetany
5·0 „	5·2 „	·263%	5·6 „	Tetany
4·5 „	6·1 „	·315%	11·2 „	Tetany relieved by extract
8·0 „	12·2 „	·381%	13·0 „	Tetany relieved by $CaCl_2$
6·3 „	12·0 „	·311%	20·0 „	Tetany relieved by $CaCl_2$ and Na_2HPO_4
5·4 „	10·0 „	·390%	14·6 „	Normal (average of series)

A fall of blood calcium below 6 mgm. per 100 c.c. is almost invariably associated with tetany. The blood phosphorus is also reduced in tetany, but not to the same extent. In the muscles the calcium values also appear to have more significance than the quantities of phosphate. Analysis of a considerable number of cases with controls show a distinct difference in the amounts of calcium present in the muscles of normal and parathyroidectomized animals. Thus in a series of analyses of normal muscle, the average amount of calcium present is 14·6 mgm. In a list of 6 cases of tetania parathyropriva with numerous controls the average is 9·5 mgm. In those parathyroidectomized animals temporarily cured of tetany there is a rise in the muscle calcium value, arising in the case of the animal injected with calcium and phosphate to 20 mgm. It may therefore be concluded that the calcium content of the muscles is reduced in tetania parathyropriva, and that the disappearance of the tetany is associated with an increase in the quantity of calcium in the muscle.

SUMMARY.

1. The alterations in the hexose-phosphate mechanism in tetania parathyropriva are secondary to the fatigue produced by the increased muscular movements occurring in this condition.

2. The calcium content of the muscle is lowered in tetany following parathyroidectomy.

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THE SUSCEPTIBILITY OF THE INFECTIVE AGENT OF THE ROUS CHICKEN-SARCOMA TO THE ACTION OF ULTRA-VIOLET RAYS.

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Received for publication August 4th, 1926.

FILTRATES of the Rous chicken-sarcoma are inactivated by most of the ordinary bactericidal agents. Rous (1912) showed that the following destroyed the activity of the tumour material: heat, 55°C. for 15 minutes; toluol, chloroform, 50 per cent. alcohol, 2 per cent. phenol. The tumour-tissue remains active for long periods when dried and for some time when kept in 50 per cent. glycerin, but both these procedures produce attenuation. Repeated freezing and thawing does not inactivate it.

Gye (1925) has used chloroform to inactivate the tumour filtrates, and when repeating his method we have found that the inactivating effect of this agent corresponds approximately with its bactericidal action on *B. coli*. Three hours at 37°C. in a closed vessel with sufficient chloroform to ensure saturation usually inactivates the tumour filtrates; *B. coli* suspended in the filtrate and treated in this manner is either very much attenuated or completely killed.

In Gye's opinion chloroform acts on the "virus" contained in the filtrate, leaving relatively intact the "specific factor." There appeared a possibility that the study of some other inactivating agent might throw some light on this question, and the well-known bactericidal effect of ultra-violet radiation suggested its use.

In this preliminary communication we have described the effects of ultra-violet radiation upon the infectivity of filtrates of the Rous chicken-sarcoma, and compared these effects quantitatively with those obtained upon some of the common pathogenic bacteria.

The question of a differential action on "virus" and "specific factor" is still under experiment and is left for a later communication.

TECHNIQUE.

Source of the rays.—A Cooper-Hewitt mercury vapour lamp has been used throughout. This particular lamp had already been standardized by one of us (P. R. P.) as far as its erythema-producing wave-lengths were concerned, and was found to have remained constant in this respect during the last 18 months. The lamp has been in use for several years, about 200 hours per annum, and appears to be very constant in its output. We have used as a standard of measurement for the present work the lethal action on *B. coli*.

The lamp circuit was arranged as in the diagram (Fig. 1), a constant power consumption of 210 watts being maintained. The current was obtained from a bank of accumulators, to avoid fluctuations which were present in the local supply from the mains. The lamp is permanently fixed in a large box of sufficient size to prevent overheating. The samples of fluid were exposed in an open glass dish with a rounded bottom (*vide* Fig. 1*b*); shielding in the angles of the sides was thus avoided. The dish was placed in a fixed position 20 cm. below the centre of the lamp and filled with the fluid under examination to a depth of 5 mm. A glass pipette (*c*), arranged so as to avoid any shielding, directed a stream of sterile air upon the surface of the fluid, producing continuous stirring.

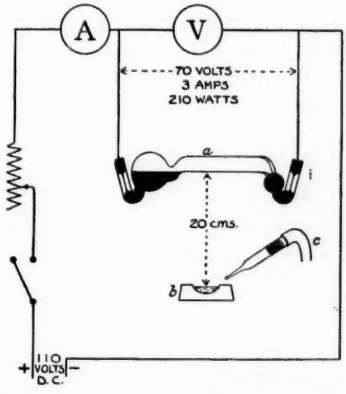

FIG. 1.—Lamp circuit and arrangement of fluid to be irradiated: *a*, lamp; *b*, dish of fluid; *c*, pipette delivering air to produce stirring. *A*, ammeter; *V*, voltmeter.

Technical considerations.—The bacteria to be treated were suspended in saline, the following points, many of which have been mentioned by other writers (Newcomer, 1917; Ellis and Wells, 1925), being considered in designing the technique:

(1) The medium in which the organisms are suspended should be fairly transparent to rays between 2000 and 3000 A.U.; ordinary broth and broth agar are almost completely opaque (amino-acids present). A trace of hæmoglobin has a considerable shielding effect; we found, for instance, that a concentration of human blood of 1 in 2500 in the saline suspensions of bacteria led to an 80 per cent. reduction in bactericidal effect.

(2) The bacterial content of the suspensions should be low enough to avoid appreciable opacity to active rays.

(3) Precautions should be taken to ensure as far as possible that the organisms are present as isolated individuals and not in clumps. Cultures on solid media are usually unsuitable for making the suspensions owing to the tendency of the organisms to remain in clumps.

It is practically impossible to obtain reliable results with certain organisms, owing to either (*a*) a difficulty in obtaining good growths from dilute suspensions, or (*b*) a tendency to form clumps.

(4) It is advisable to keep the suspensions in motion during exposure so as to ensure uniformity of effect.

Effects of density of bacterial suspensions.—An increase in the number of organisms in the suspensions used influences the apparent lethal dose in two ways: (*a*) it increases the opacity of the medium as a whole to the effective rays; (*b*) it increases the number of surviving organisms in suspensions treated with doses which have just failed to produce complete extinction.

In any collection of bacteria, individual susceptibility to ultra-violet rays varies within a considerable margin of dose, this variability presumably depending upon age and general state of vitality of individual organisms. The

curve in Fig. 4 is obtained by charting percentage survival after varying doses. There is a destruction of the majority of the organisms with a comparatively small dose; a few per thousand, however, survive a much longer treatment, possibly on account of their positions in the centres of small clumps, which no doubt exist even in the most carefully prepared suspensions.

Apart from any shielding effects, the actual number of organisms surviving at any particular stage of the treatment should be roughly proportional to the number of living organisms in the initial suspensions.

Table I shows the effect of the number of bacteria in the suspensions upon the number surviving after radiation.

The first 4 suspensions show the combined effects of increase of opacity and increase in numbers; the last sample, a mixture of living and killed organisms, shows the effect of opacity by itself.

It is obvious from the above considerations that if the term "lethal dose" implies complete sterilization of the suspension, it will vary with the initial bacterial content. Only by taking a convenient arbitrary density of suspension can we obtain constant results; as long as the bacterial content is kept sufficiently low, however, there is a fairly wide margin within which uniform results can be obtained.

TABLE I

Nº of living B. Coli per c.c.	U-V Dose (in units) 7	10	15	22½	34	50
64×10^3	*320	0+	0-			
50×10^4		1,000	0+	0-		
50×10^5			300	0+	0-	
50×10^6				700	6	0+
50×10^6 + 50×10^6 (killed)		2,000	500	1	0-	

* First column = numbers in untreated suspensions. figures = number of colonies in agar. + = positive broth tube. − = negative broth tube.

A more accurate comparison between the resistances of various organisms can, in our opinion, be made by adopting an end-point corresponding to a reduction to a low figure, say 1/10,000, than by aiming at complete sterilization.

In the case of the filtrates of the Rous tumour the reduction of the quantity of active agent can only be estimated by comparison with that obtained by progressive dilution.

During our experiments the samples of filtrate used have varied in virulence, but the smallest dose which has produced a positive result up to the present has been 0·5 c.c. of a 1/1000 dilution in saline. It is fairly safe to assume, therefore, that an attenuation to a 1/10,000 part would inactivate the filtrates we have been using. This degree of reduction is of the same order as that which is attained by the end-point for the bactericidal effect, so that the comparison which will be made between lethal action on bacteria and on the tumour agent may, we think, be regarded as a fair one.

Bacteriological technique.—Young broth cultures, about 18 hours old in the case of rapidly growing organisms, were diluted in saline so that the bacterial content did not exceed about 100,000 per c.c. of living organisms. The suspension was water-clear, and we were satisfied that there could be no effective shielding of one organism by another. In testing the fluid 0·5 c.c. was added to 4·5 c.c. of broth and 0·5 c.c. of this added to melted agar.

After incubation a colony count was made and growth or otherwise in the broth-tube noted.

RESULTS WITH BACTERIA.

With the above procedures we have been able to obtain very constant effects. Table II shows the results with a particular strain of *B. coli* tested on various dates with different doses of radiation.

For the purposes of this investigation the average dose which just fails to kill *B. coli*,* suspended in saline in a concentration of about 100,000 organisms per c.c., has been called 10 units. This is an exposure of 26 seconds with our lamp worked under the conditions described, and happens to be almost exactly

TABLE II

U·V Dose---	0		7·5	8·5	10	12	15
17/3/26	B.Coli	∞		100		0-	
22/4/26	"	3,000			0+		0-
21/5/26	"	∞			2		0-
30/6/26	"	∞			0+		0-
12/7/26	"	∞			16		0-
22/7/26	"	6,400	320		0+		0-

Dose expressed in units (*vide* text). Numbers = colonies in agar. ∞ = large numbers (10–100 thousand). + = positive broth tube. − = negative broth tube.

TABLE III

U·V Dose---	0	8·5	10	12	15	17	28	58
B.Coli	∞	120		1		0-		
B.Coli	∞	100		0-				
B.Typhosus	∞	3		0-				
B.Typhosus	∞	1		0-				
B.Para-T A	∞	1		0-				
B.Para-T B	∞	68		0-				
B.Para-T C	∞		12		0-			
B.Prodigiosus	∞		2		0-			
Staph.Aureus	∞			0+		0-		
M.Melitensis	∞		300		0-			
Yeast	43,000				16,000		1,000	0-

Figures apply as in Table II.

1/10th of the exposure required to produce an erythema, appearing in 2 hours, on the arm of P. R. P.

The organisms examined include strains of *B. coli*, *B. typhosus* and *para-typhosus*, staphylococcus, *B. prodigiosus*, *M. melitensis*, and a yeast.

With the exception of the yeast, the organisms tested showed very little difference in susceptibility to irradiation (Table III). The vanishing point for colonies is obtained with a dose of 9–12 units (24–30 seconds), and 15 units (about 40 seconds) gives a negative broth-tube.

EFFECTS ON TUMOUR FILTRATE.

Having satisfied ourselves as to the uniformity of effect upon *B. coli* and the various pathogenic bacteria examined, we next proceeded to investigate the Rous tumour. Throughout the work we have used a filtrate obtained by

* Absence of colonies in agar, but positive broth-tube.

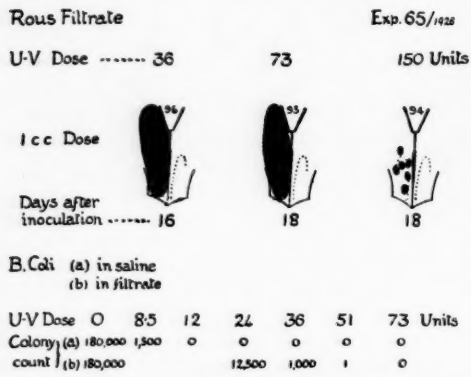

FIG. 2.—Showing comparison between the effects of ultra-violet radiation on *B. coli* and on filtrate of Rous sarcoma. *a*. Lethal dose on *B. coli* suspended in saline is about 12 units. *b*. Control on *B. coli* suspended in filtrate shows a lethal dose of about 51 units. Diagrams show anterior aspect of chicken's thorax; birds inoculated into right breast. No. 94 shows attenuation of active agent by a dose of 150 units.

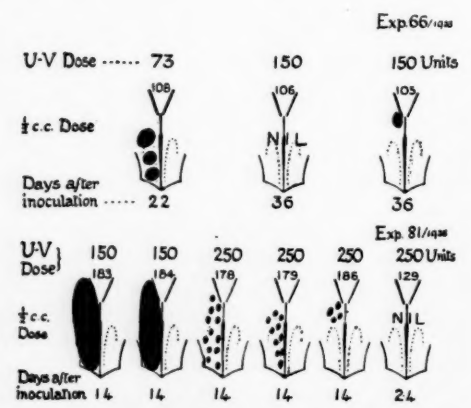

FIG. 3.—Showing further results of irradiation on infectivity of tumour filtrate. Chickens Nos. 178, 179 and 186 show the results of a filtrate still active after a dose of 250 units—five times the control lethal dose for *B. coli*.

grinding the fresh tumour with sand, mixing with normal saline in the proportions of 2·5 gm. of tumour to 100 c.c. of saline, and filtering by gravity through paper pulp and sand. The resulting fluid is nearly water-clear and almost colourless; it contains, however, an appreciable quantity of coagulable protein, and absorbs a considerable proportion of the bactericidal wave-lengths of ultra-violet rays. It was necessary to obtain a fairly exact quantitative estimate of the amount of effective radiation which would reach particles suspended in this fluid. This was done by suspending *B. coli* in a sample of the filtrate and finding the lethal dose under these conditions. It will be seen from Fig. 2 that in filtrate the lethal exposure is approximately five times that necessary in saline. The lethal dose for coli in filtrate is therefore, according to our scale, about 50. If care was taken to avoid the presence of hæmoglobin in the filtrate we found that the "coli dose" in different samples varied only between 50 and 60 units. A control of this sort containing the bacilli was, however, put up with every batch of filtrate tested.

Samples of filtrate were now given various lengths of exposure and injected into the pectoral muscles of fowls.

Figs. 2 and 3 illustrate the effects obtained.

When active filtrate is injected into the pectoral muscles of a fowl the tumour always appears as multiple foci, and can be seen in the early lesions as opaque spots on the fascial surfaces of the muscles. In the case

of a large dose these foci coalesce early to form a more or less diffuse mass of infiltration; when the fluid is diluted or attenuated the foci remain for some time as isolated spots, which may finally fuse into a nodular mass. The effects obtained have been somewhat variable, depending probably on the virulence of the different samples of filtrate used. It is quite certain, however, that some samples of filtrate will remain active, though attenuated, even after a dose of 250 units—that is, 5 times the lethal dose for coli in filtrate.

It is known that ultra-violet radiation will inactivate complement, and we have worked out the dose on a sample of guinea-pig serum. Fig. 4 shows the comparative effects of the rays upon *B. coli*, complement and Rous-tumour filtrate.

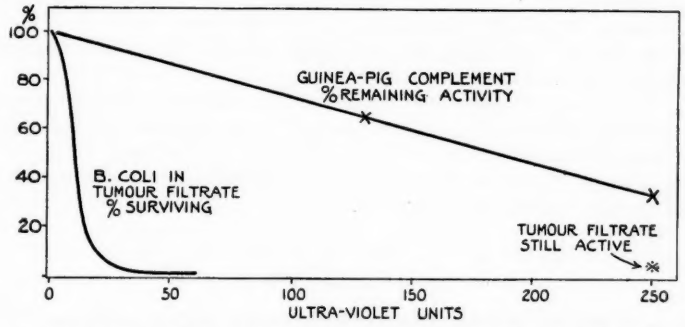

FIG. 4.—Curves showing comparison between action of ultra-violet radiation on *B. coli*, complement and filtrate of Rous sarcoma. Curves of *B. coli* and complement show percentage attenuation after varying doses. * Marks point at which filtrate is still active though attenuated.

DISCUSSION.

The active agent in the tumour filtrate will survive a dose of ultra-violet radiation at least 5 times that necessary to kill most of the ordinary pathogenic bacteria.

It seems probable that the size of the particles may be the factor in determining this difference in susceptibility. We have not detected any difference in action on large and small bacteria; *M. melitensis* is as susceptible as *B. coli*, but neither of these bacteria is small in comparison with the effective wave-lengths employed (0·2–0·3 μ).

A very much larger organism, a yeast several thousand times the volume of *B. coli*, showed a marked resistance to irradiation, as has been noted by other observers. The comparative insusceptibility in this case probably depends on lack of penetration of the rays to the deeper parts of the cell. The yeast showed a phenomenon which was not observed in the case of the bacteria, namely a marked retardation in the growth of the colonies surviving irradiation. The controls showed well-developed colonies in about 18 hours, while the cultures of irradiated samples showed no visible growth at this time,

but with sub-lethal doses numerous colonies developed considerably later. This effect might well be interpreted as due to a superficial damage to the organism, not sufficient to kill, but enough to retard reproduction for a time. Passing to the active agent in the filtrate at the other end of the dimensional scale, the particle is quite possibly sufficiently small in comparison with the longer bactericidal wave-lengths to be unaffected by this part of the spectrum. It may be possible to gain some information on this point by using only the shorter wave-lengths in the bactericidal range.

SUMMARY.

(1) A technique for obtaining quantitative lethal effects upon organisms with ultra-violet radiation is described.

(2) It is shown that the various types of bacteria tested were equally susceptible to irradiation.

(3) A comparison is made between the lethal effects of the rays on ordinary bacteria and upon the active agent in the Rous chicken sarcoma, and it is shown that the latter is much less susceptible.

(4) It is suggested that the relative insusceptibility of the agent in the tumour filtrates is due to its small size.

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THE *RÔLE* OF THE RETICULO-ENDOTHELIAL SYSTEM IN THE REGULATION OF THE NUMBER OF PLATELETS IN THE CIRCULATION.

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Received for publication August 16th, 1926.

THE researches of recent years have added considerably to our knowledge of the life-history and functions of the platelet, and yet we are still without precise information as to the normal mechanism for the destruction of these elements of the blood when they become effete. The formed elements of the blood normally enter the circulation in a mature state, play their part, and when no longer serviceable, are removed and destroyed. Their sojourn in the blood is of more or less short duration, and in the case of the platelet—a fragile disc of cytoplasm without a nucleus—it is hardly conceivable that its life in the circulation should be anything but short. Since their numbers in the circulation remain at a constant level, a balance must be struck between production and destruction, and it follows from this that any factor which brings about an increase in the number of platelets can only do so in one of three ways:

(1) By increasing production.
(2) By decreasing destruction.
(3) Or by both.

It has been definitely established, both in man and in other animals, that removal of the spleen is followed by a rapid increase in the number of circulating platelets. In the normal guinea-pig this procedure was found to double the platelet count in 48 hours (Bedson, 1924), and the same holds true for the rabbit, as will be seen from experimental data to be given later. This effect of splenectomy has received two explanations:

(1) That the spleen normally removes effete platelets from the circulation and destroys them.
(2) That the spleen exerts some inhibitory action on platelet production.

In support of the first of these two hypotheses is the richness of the spleen in platelets. This can be demonstrated by the microscopic examination of smear preparations of the spleen made with a technique appropriate for the conservation of platelets, and the hypothesis finds still further support in the observation (Bedson and Johnston, 1924) that an anti-spleen serum may contain platelet antibody much in excess of what could be accounted for by the amount of blood contained in that organ, assuming the platelets to be present in the same proportion as in the circulating blood. Recently Meyers, Maingot and Gordon (1926) have recorded platelet counts made on the blood of the splenic vein and artery in a case of essential thrombopœnia coming to operation. It was distinctly lower in the splenic vein (56,000 against 168,000),

and though the conditions under which these counts were made would not lend themselves to accuracy, the findings would appear to indicate that the spleen, at any rate under certain conditions, is capable of destroying platelets. Alrutz, Norbell and Piette (1926) claim to have demonstrated excessive destruction of platelets in the endothelial cells of a spleen removed in a case of thrombopœnic purpura, which would further support this view. If the spleen removes and destroys platelets it probably does so by means of its phagocytic cells, which form so important a part of the reticulo-endothelial system. These cells throughout the body have the power of picking out particulate matter introduced into the circulation—in fact they can be so packed with such material (trypan blue, carmine, carbon, colloidal silver) as to temporarily upset their functions, and this so-called "blockade" of the reticulo-endothelium has been employed in investigating the *rôle* played by this system of cells. (Jungeblut and Berlot, 1926. The papers by these authors contain a good bibliography of the subject.) It occurred to the author that in this blockade experiment a method was available for elucidating the *rôle* of the spleen in maintaining the platelet balance, and the results of the experiments carried out with this object form the subject of the present communication.

EXPERIMENTAL DATA.

Technique.—The platelets were counted by the indirect method already described by the author (Bedson and Zilva, 1923). Indian ink (Gurr), diluted 1 in 5 with saline and sterilized by autoclaving, was used for blocking the reticulo-endothelial cells. Rabbits were used exclusively as the experimental animal, receiving four to five intravenous inoculations of 2·0–4·0 c.c. of the above suspension of ink at intervals of one to three days. Splenectomy was carried out through an incision starting about an inch from the vertebral spines and running parallel to and a little below the costal margin.

Effect of Splenectomy on the Platelet Count in the Normal Rabbit.

The spleens from two normal rabbits were removed, the platelets being counted before and at intervals after operation. These counts are given in Table I.

TABLE I.

Date.	Rabbit B. 8.			Rabbit B. 10.		
	R/P.	Red corpuscles.	Platelets.	R/P.	Red corpuscles.	Platelets.
8 . iv . 26	6·2	5,552,000	895,000	8·2	6,112,000	745,000
9 . iv . 26		Splenectomy			Splenectomy	
10 . iv . 26	4·0	5,940,000	1,482,000	5·0	5,760,000	1,132,000
15 . iv . 26	4·2	5,008,000	1,192,000	4·6	4,536,000	986,000
23 . iv . 26	5·2	5,760,000	1,109,000	—	—	—
30 . iv . 26	5·4	6,376,000	1,180,000	—	—	—
21 . v . 26	6·7	6,800,000	1,015,000	7·1	6,496,000	915,000
16 . vi . 26	6·7	6,344,000	947,000	7·7	6,680,000	867,000
29 . vi . 26	6·5	5,960,000	917,000	7·0	6,640,000	920,000

R/P = Red corpuscle : platelet ratio.

It will be seen that in both cases removal of the spleen was followed by a considerable rise in the platelet count, which was most marked 24 hours after operation, gradually falling again to approximately the normal level in a little over two months.

Effect of "Blockade" with Indian Ink on the Platelet Count.

This was the next point investigated, and, as will be seen from the counts given in Table II, the rise in the platelet count approximates to that produced by splenectomy.

TABLE II.

Rabbit B. 1.					Rabbit B. 3.				
Date.	Intravenous inoculation of 20 % Indian ink.	R/P.	Red corpuscles.	Platelets.	Date.	Intravenous inoculation of 20 % Indian ink.	R/P.	Red corpuscles.	Platelets.
9 . iii . 26	—	8·4	6,296,000	748,000	12 . iii . 26	—	8·0	6,960,000	870,000
11 . iii . 26	4·0 c.c.	—	—	—	,,	3·0 c.c.	—	—	—
12 . iii . 26	3·0 c.c.	—	—	—	13 . iii . 26	—	6·8	7,216,000	1,061,000
13 . iii . 26	—	6·4*	5,240,000	810,000	15 . iii . 26	—	6·3	6,840,000	1,085,000
15 . iii . 26	2·0 c.c.	—	—	—	,,	2·0 c.c.	—	—	—
16 . iii . 26	2·0 c.c.	—	—	—	16 . iii . 26	2·0 c.c.	—	—	—
17 . iii . 26	—	5·2	6,384,000	1,227,000	17 . iii . 26	—	6·0	6,400,000	1,066,000
,,	3·0 c.c.	—	—	—	,,	3·0 c.c.	—	—	—
18 . iii . 26	—	4·0	5,520,000	1,380,000	18 . iii . 26	—	6·1	6,800,000	1,114,000

* Had litter during night, 12th to 13th March.

It will be seen that in rabbit B. 1 the count rose gradually to a maximum, whereas in B. 3 the first inoculation produced an immediate rise and that further inoculations failed to influence the count very much. A third rabbit was submitted to the same procedure in order to see how long the rise produced in the platelet count by blockade lasted; these findings are shown in Table III.

TABLE III.

Rabbit B. 19.				
Date.	Intravenous inoculation of 20 % Indian ink.	R/P.	Red corpuscles.	Platelets.
12 . vii . 26	—	6·2	6,320,000	1,020,000
,,	3·0 c.c.	—	—	—
13 . vii . 26	3·0 c.c.	—	—	—
14 . vii . 26	3·0 c.c.	—	—	—
15 . vii . 26	3·0 c.c.	—	—	—
16 . vii . 26	3·0 c.c.	—	—	—
19 . vii . 26	—	4·1	8,084,000	1,971,000
21 . vii . 26	—	6·0	6,440,000	1,073,000
22 . vii . 26	—	6·1	6,640,000	1,088,000
23 . vii . 26	4·0 c.c.	—	—	—
26 . vii . 26	—	7·3	6,960,000	953,000

It will be seen from the above figures that five days after the last inoculation the platelet count, which had risen from 1,020,000 to 1,971,000, had fallen again to the pre-inoculation figure, and that a further inoculation of Indian ink was no longer productive of a rise. This points to the effect of blocking as being quite transitory, and suggests that the functions of these cells are for a time upset owing to their having to deal with an overwhelming quantity of abnormal material. Later, though the ink particles are still present in these cells, they would appear to readjust themselves to the new conditions and resume their normal functions. Similar conclusions as to the temporary nature of the upset in function produced by blockade have been arrived at by Jungeblut and Berlot (*loc. cit.*) in their work on the part played by the reticulo-endothelial system in the production of antibodies.

Effect of Splenectomy in a Blockaded Animal.

Should the spleen be destroying platelets in virtue of its possession of a large complement of reticulo-endothelial cells, then an animal which has had these cells temporarily upset by blocking with Indian ink, as is shown by a rise in the platelet count, should show no further increase in the number of these elements in the circulation on removal of the spleen. This argument assumes that the upset in function produced by blocking is temporarily complete as far as platelet destruction is concerned. In Table IV are given the findings in two rabbits in which the platelet count was raised by the inoculation of Indian ink and which then had their spleens removed.

TABLE IV.

Rabbit B. 5.					Rabbit B. 6.				
Date.	Intravenous inoculation of 20 % Indian ink.	R/P.	Red corpuscles.	Platelets.	Date.	Intravenous inoculation of 20 % Indian ink.	R/P.	Red corpuscles.	Platelets.
7.iv.26	—	7·5	6,256,000	834,000	7.iv.26	—	7·5	6,400,000	853,000
,,	3·0 c.c.	—	—	—	7.iv.26	3·0 c.c.	—	—	—
8.iv.26	3·0 c.c.	—	—	—	8.iv.26	3·0 c.c.	—	—	—
9.iv.26	3·0 c.c.	—	—	—	9.iv.26	3·0 c.c.	—	—	—
10.iv.26	3·0 c.c.	—	—	—	10.iv.26	3·0 c.c.	—	—	—
12.iv.26	3·0 c.c.	—	—	—	12.iv.26	3·0 c.c.	—	—	—
13.iv.26	—	4·7	6,000,000	1,276,000	13.iv.26	—	4·0	5,384,000	1,346,000
	Splenectomy					Splenectomy*			
14.iv.26	—	4·2	5,472,000	1,303,000	14.iv.26	—	4·2	3,704,000	882,000
27.iv.26	—	5·3	6,200,000	1,170,000	19.iv.26	—	3·3	4,864,000	1,474,000
18.v.26	—	6·9	6,278,000	909,000	27.iv.26	—	5·4	5,288,000	979,000
15.vi.26	—	6·4	6,480,000	1,012,000	18. v.26	—	7·3	6,464,000	885,000

* The operation in this animal was attended with a considerable loss of blood. The spleen in the injected animal is swollen and tense and much more difficult to remove than in the normal animal.

In both animals the removal of the spleen did not bring about any further increase in the number of platelets in the blood. The fall recorded in B. 6 24 hours after the operation was due to the hæmorrhage and this is borne out by the red-cell figure. That no increase in platelets did result from splenectomy is shown conclusively by the red-cell platelet ratio, which remained steady.

Effect of Blockade in a Splenectomized Animal in which the Platelet Count has fallen to Normal again.

It remained to be seen whether in a splenectomized animal which has compensated for the loss of that organ (return of platelet count to normal figures), the blocking of the reticulo-endothelial system results in a rise in the number of circulating platelets. In the rabbit this compensation requires about two months to be established; in the guinea-pig it is complete in a month (Bedson, *loc. cit.*). The two rabbits B. 8 and B. 10, the platelet counts of which before and after splenectomy are given in Table I, were given a series of four intravenous inoculations of Indian ink, commencing on 1 . vii . 26, when the platelet count has fallen to approximately a normal level after splenectomy. The result of these inoculations on the platelet count can be seen from the data given in Table V.

TABLE V.

	Rabbit B. 10.				Rabbit B. 8.			
Date.	Intravenous inoculation of 20 % Indian ink.	R/P.	Red corpuscles.	Platelets.	Intravenous inoculation of 20 % Indian ink.	R/P.	Red corpuscles.	Platelets.
29 . vi . 26	—	7·0	6,440,000	920,000	—	6·5	5,960,000	917,000
1 . vii . 26	3·0 c.c.	—	—	—	3·0 c.c.	—	—	—
2 . vii . 26	3·0 c.c.	—	—	—	3·0 c.c.	—	—	—
3 . vii . 26	3·0 c.c.	—	—	—	3·0 c.c.	—	—	—
5 . vii . 26	3·0 c.c.	—	—	—	3·0 c.c.	—	—	—
6 . vii . 26	—	4·5	6,480,000	1,440,000	—	4·9	6,160,000	1,257,000

In both cases blockade of the reticulo-endothelial system resulted in a rise in the platelet count comparable to that obtained before removal of the spleen. It would appear, then, from the result of this experiment, that in the absence of the spleen, the maintenance of the platelet balance is taken on by some other part of the reticulo-endothelial system.

DISCUSSION.

It is established that splenectomy produces a considerable increase in the number of circulating platelets, this increase attaining its maximum within the first twenty-four hours after operation, followed by a gradual return to normal level. Evidence has been produced in this paper to show that when the cells

of the reticulo-endothelial system are temporarily upset by blocking with inert particles such as carbon, an equally marked rise in the platelet count occurs, though it is slower in reaching its maximum. Further, it has been shown that the rise following splenectomy cannot be superimposed on the rise following the injection of indian ink, from which it follows that in all probability the rise in each case is due to the same cause, viz. putting out of action, temporarily or permanently, cells of the reticulo-endothelium. In the blockade experiments a very considerable portion of the reticulo-endothelial system is concerned and presumably suffers equally from the temporary upset in functions, whereas in splenectomy only a portion of this system of cells is removed. However, since splenectomy is not productive of a further rise in the platelet count in a blockaded animal, it seems reasonable to suppose that normally it is that part of the reticulo-endothelial system residing in the spleen which is mainly responsible for maintaining the platelet balance. That this is a function of the reticulo-endothelial cells, and not essentially one of the spleen, is shown by the fact that the balance is soon restored after having been upset by splenectomy, and that it can then be upset again by blocking the remaining reticulo-endothelial cells in the usual way. How, then, does this system of cells maintain the platelet count at a constant level? If one assumes that they exert some inhibitory action on platelet production, where are the used-up platelets destroyed, and by what mechanism and how is one to account for the presence of such large numbers of platelets in the blood sinuses of the spleen? The reticulo-endothelial cells, are essentially phagocytic, and it seems much more reasonable to suppose that the effete platelets are held up in the sinuses of the splenic pulp, just as in the case of the red blood-corpuscles (Barcroft, 1926), there to be destroyed by those phagocytic cells in which the spleen pulp is so rich. All the available evidence supports this explanation, whereas the alternative hypothesis remains in the realm of pure conjecture. Towards the completion of this investigation, Koster (1926) published the results of his experiments on the relation of the reticulo-endothelial system to the platelet. Although his findings in a measure support those of the author, they appear to be open to certain criticisms. The direct method of counting platelets is inaccurate, for the reason that it entails the bringing of the blood in contact with a surface to which platelets will adhere before mixing it with the anticoagulant, incurring an inevitable loss of platelets. This method was abandoned by the author some years back for this reason, and probably accounts for the abnormally low platelet counts recorded by Koster in normal guinea-pigs. Koster blocked the reticulo-endothelial cells in guinea-pigs by two inoculations, 5·0 c.c. each, of a 0·5 per cent. suspension of trypan blue given subcutaneously, and yet after this procedure, which could hardly be expected to produce an efficient blocking of these cells, the platelet count rose to over four times the normal level. It would appear that here both accelerated production and lessened destruction would have to be called in to explain the enormous increase. When the count was returning to normal after the initial blockade, Koster found that a further inoculation of trypan blue was not followed by a rise in the platelet count, and from this observation deduces that there must be some other means of platelet destruction than phagocytosis. It seems, however, more reasonable to suppose that the effect of blockade is only temporarily effective,

and does not run parallel with the persistence of the injected particles in the reticulo-endothelial cells. This view finds support in the work of Jungeblut and Berlot already referred to.

The bearing of this work on the treatment of essential thrombopœnia is worthy of consideration. Apparently the destruction of abnormal or effete platelets is a function of the reticulo-endothelial system, and normally this takes place in the spleen. However, in the absence of the spleen, the remaining portion of this system of cells can assume this *rôle*. At present we are ignorant as to the essential pathology of this condition. Is the essential lesion one of the platelets, or is it some abnormal condition of the reticulo-endothelial system, and if so, is this change confined to that portion contained in the spleen? Kaznelson holds the latter view, and in support of it advances his observations made on the bone-marrow of cases of essential thrombopœnia obtained both post-mortem (Kaznelson, 1919) and during life by sternal puncture (Weiner and Kaznelson, 1926). He finds the megakaryocyte both normal in numbers and appearance, and considers them functionally active cells, capable of producing normal platelets. It should be pointed out, however, that though there is an abundance of circumstantial evidence in favour of the megakaryocyte being the parent cell of the platelet, there is no direct evidence, other than morphological, in support of this widely accepted view. On the other hand, Rockwood and Sheard (1926), by means of instantaneous photomicrography, claim to be able to demonstrate a qualitative change in the platelet in essential thrombopœnia, a change which was not found in the platelets in a case of pernicious anæmia associated with a low platelet count and purpura. Most observers are agreed that the spleens removed at operation in cases of essential thrombopœnia show a hyperplasia of the endothelial phagocytes, but is this primary or secondary? Certainly the clinical results obtained by splenectomy in this condition would point to the spleen as the seat of the essential pathological change, since on its removal the hæmorrhagic state disappears, and the patient may remain in good health for as long as ten years. But it must be pointed out that it is only the chronic cases which are considered suitable for operation, and it is not unknown for these to clear up spontaneously and remain well for considerable periods of time.* It will not be until we have methods available for determining qualitative changes in the platelet that we shall arrive at a proper understanding of the purpuras, and be able to say whether splenectomy can be expected to provide a permanent cure or only a temporary amelioration in a given case.

CONCLUSIONS.

(1) The temporary upset in function of the reticulo-endothelial cells produced by filling them with carbon particles is associated with a considerable increase in the number of platelets in the circulation.

(2) Removal of the spleen when blockade of the reticulo-endothelial system

* In addition to this some of the cases treated by splenectomy have relapsed, and though it might be contended that these were not true cases of essential thrombopœnia, this would merely emphasize the fact that the use of this operative procedure is not as yet based on scientific fact.

is at its height of functional activity is not productive of any further rise in the platelet count.

(3) The spleen would appear to regulate the number of platelets in the circulation by means of its reticulo-endothelial cells. In view of the essentially phagocytic nature of these cells, it seems reasonable to suppose that they take up and destroy the platelets which are no longer of use.

(4) When the spleen is removed, this function is taken on by some other portion of the reticulo-endothelial system.

This work has been carried out under the auspices of the Freedom Research Fund of the London Hospital.

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THE PATHOLOGICAL EFFECTS OF HYPNOTIC DRUGS UPON THE CENTRAL NERVOUS SYSTEM OF ANIMALS.

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From the Research Laboratories, Hollymoor, Birmingham.

Received for publication July 30th, 1926.

THIS investigation was undertaken two years ago at the suggestion of Sir Frederick Mott, who, following a discussion with Sir William Willcox, expressed the desirability of investigating the histological and histochemical changes which possibly might occur in the central nervous system following the action of certain hypnotic drugs.

Accordingly a series of experiments upon animals was undertaken.

METHODS OF INVESTIGATION.

Animals employed.—The animals used were cats and monkeys. Cats were chosen because of their availability in litters, their carnivorous nature and their comparatively high grade of intelligence, which facilitated clinical observation; monkeys were selected on account of the superior development of the nervous system, which presented a closer approximation to the human subject.

Drugs used.—The drugs administered belong to the two groups:

Type 1. *Sulphone group:* $(R.SO_2)_2 CR_2$.
Examples: Sulphonal, trional.

Type 2. *Barbitone group:* $CO(NH\,CO)_2 CR_2$.
Examples: Veronal, dial, luminal, soneryl.

Administration and doses of drugs.—The drugs were given by mouth, in minced meat or suspended in a little milk in amounts of $2\frac{1}{2}$ to 5 gr. daily for cats and 5 to 10 gr. daily for monkeys.

The duration of the administration varied from a few days to several weeks, so that investigation could be made of both recent and cumulative effects.

The time taken to recover from the symptoms upon cessation of drug treatment was observed, and the condition of the central nervous system was correlated with the period of recovery. In one series of experiments the general effects of the drugs on the growth and development of kittens taken from litters were observed by weighing at regular intervals and comparing with normal controls from the same litter.

After the intended administration of the drug the animal was killed, and specimens of the organs were suitably preserved for histological and histochemical examination, together with normal tissue preserved and treated in an exactly similar manner from control animals.

In one experiment, 5 gr. of veronal in a finely divided state, suspended in olive oil, were injected daily subcutaneously into a monkey; in another experiment, 10 gr. of urethane ($CO\langle^{NH_2}_{OC_2H_5}$) in aqueous solution were injected daily, also into a monkey.

CLINICAL OBSERVATIONS.

The record of the weight of the animals showed that the drugs sulphonal and trional caused a marked disturbance of nutrition in growing animals, cats of 1500 gm. losing 200–400 gm. in three weeks, whilst normal animals gained about 150 gm. in the same period.

The barbitone series of drugs caused a less degree of disturbance, veronal and dial showing least; but with luminal a greater loss of weight occurred. Untreated controls gained 800 to 900 gm. during a period in which the veronal-fed animals gained only 400 gm. These figures were obtained on cats from the same litters. The treatment of cats with sulphonal and trional resulted in every case in the occurrence of ill-health; their coats lost lustre and became rough, the animals did not clean themselves from accidental dirt and appeared dull and apathetic. They soon showed loss of appetite and developed marked ataxia, especially noticeable in the hind limbs; there was also progressive atonia leading to a state of exhaustion. The general perceptions of the animals became quite dulled. In the earlier stages of the treatment the animals walked unsteadily on a broad base, easily fell over, could not jump correctly, and had difficulty in alighting upon their feet when allowed to fall from a height. There were also present tremulous motions of the head and limbs resembling an "intention tremor"; these were most obvious when the animals attempted to take food. During intensive treatment these tremors were much more pronounced; the tendon-jerks were much exaggerated. The barbitone group of drugs showed similar but less pronounced exhaustive effects, and it was found that veronal and dial could be given to cats for many days without appreciably impairing the general well-being of the animals. When the doses were slowly increased, the animals seemed to acquire some degree of tolerance to moderate doses of drugs of this group.

Sleep was not induced in cats by any of the drugs tested; when large doses were given, they developed a comatose condition in which they died. In some cases we thought it possible that a short period of sleep might have preceded the comatose state, but the margin between sleep and coma was so slight as to make such observations very difficult.

The action of both groups of narcotics upon monkeys was similar in the production of atonia, muscular weakness and ataxia, but a condition resembling normal sleep was readily produced in these animals, although the relative quantities of drugs necessary were very much greater in proportion to those prescribed for man.

Monkeys of five pounds weight slept 6 to 24 hours after having received 5 to 10 gr. of sulphonal or veronal, provided they had previously taken small doses for some time. Following cessation of the drugs for a few days they became normal and showed no permanent clinical effects.

Cats made a rapid recovery from an advanced state of drug treatment, and after an interval of 10 to 14 days showed no observable impairment of normal activity and intelligence. During the administration of the drugs an attempt was made to observe the effect of the hypnotic upon the basal metabolism of the cats in various stages of the treatment. They were enclosed in a sealed glass vessel of 30 litres capacity for about 20 minutes and samples of the contained air were withdrawn before and after the experiment. The carbon dioxide and oxygen content of these samples was obtained by analyses in a Haldane gas analysis apparatus and the gas exchange calculated.

It was found, however, that movements of the animals were sufficient to prevent any accurate comparison of the metabolic rates. However, it was observed that the animals in a state of advanced atonia had a metabolism which was very much less than that of the normal quiet animals; and, as might have been expected, we found that cats in semi-comatose condition had a metabolism which was only 50% of the figure obtained with the average quiet cat.

Tests were made for albumen and blood-pigment in the urine of the treated animals, usually with negative results.

Albumen and a trace of blood was found in the urine of a cat dying from soneryl; and the urine of a monkey after prolonged sulphonal feeding contained a little albumen but without blood-pigment.

EXPERIMENTAL.

Table I records briefly the particulars of experiments carried out on a number of animals, the tissues of which were examined, giving results described in the text.

Effects of the Drugs on the Organs of the Animals.

Method of histological control.—Normal animals were reared under the same conditions as the drug-treated animals, and were killed by the same method; their tissues were fixed in the same fixatives for exactly similar periods. The fixed tissues were then either cut as frozen sections of similar thickness, or mounted together in the same block of paraffin or celloidin; sections containing the tissues of normal and treated animals side by side were thus stained for comparison.

Effects on the organs other than the central nervous system.—In the liver and kidney of the treated animals some fatty infiltration and degeneration was often found, but since the control animals usually showed a certain amount of similar changes, the investigation of this condition was not continued.

No macroscopic lesions were observed in the other viscera of the animals.

Changes in the central nervous system.—Histological examination of the formalin-fixed specimens of every animal which had received the drugs for a period of many days showed a quantity of an organic deposit scattered throughout the nervous system. The substance was found in the cord and

TABLE I.—*Record of Experiments on Animals.*

Experiment No.	Animals and approximate weight.	Drug administered.	Method of administration.	Daily dose of drug (grains).	Period of treatment.	Total quantity of drug given (grains).	Days for recovery before killing.	Further notes on the experiment.
1*a*	Cat, 1500 gm.	Veronal	By mouth	5	1 dose	5	0	Killed 18 hours after giving dose.
1*b*	Cat, 1600 „	Sulphonal	„	5	„	5	0	Killed 18 hours after dose together with a normal control.
1*c*	Cat, 600 „	Veronal	„	5	„	5	0	Killed 18 hours later whilst in a comato sestate. Tissues fixed and stained with a normal control and those of 3*b*.
2	Cat, 2000 „	Veronal	„	5	5 days	25	0	Animal was killed a few hours after the last dose.
3*a*	Cat, 2000 „	Veronal	„	5	9 „	45	0	—
3*b*	Cat, 600 „	Veronal	„	2	10 „	20	0	Killed with normal control and animal 1*c* for comparative examination.
3*c*	Cat, 800 „	Soneryl	„	½–2½	14 „	18	0	Died in a comatose condition after last dose.
4*a*	Cat, 2000 „	Veronal	„	1–2 5 10	5 weeks 80 days About 10 occasions	600	0	Gained 460 gm. in weight. The animal was ataxic over a long period.
4*b*	Cat, 1700 „	Luminal	„	½–1½	10 weeks	50	0	Lost 200 gm. in weight.
4*c*	Cat, 1400 „	Dial	„	½–1	7 „	35	0	Gained 100 gm. in weight.
4*d*	Cat, 1500 „	Veronal	„	2–4	5 „	120	0	—
4*e*	Cat, 1600 „	Trional	„	1½–2½	4 „	50	0	Lost 380 gm. in period.
4*f*	Cat, 1800 „	Sulphonal	„	1–2½	4 „	50	0	Lost 270 gm. in this period.
5*a*	Cat, 1500 „	Veronal	„	2½–5	48 days	170	21	No clinical signs of impairment of health when killed.

5*b*	Cat, 1200 gm.	Veronal	By mouth	5	10 days	50	5	The three animals in experiments 5*b*, 5*c*, 5*d* were killed together.
5*c*	Cat, 1200 „	Veronal	„	5	10 „	50	10	
5*d*	Cat, 1300 „	Veronal	„	5	10 „	50	0	
X	Cat, 2500 „	No drug given	—	—	—	—	—	—
6*a*	Monkey	Sulphonal	By mouth	5	6 weeks 32 days	150	0	Slept on a number of occasions after receiving dose (28 hours on one occasion). Muscles weak on awakening, but generally recovered in 3 days. Health and appetite was maintained during the period. Killed after a dose of 10 gr. on previous night.
6*b*	Monkey	Veronal	„	5	52 days	160	0	Sleep resulted from the administration of drug on several occasions.
7	Monkey	Veronal	„	4·5	20 „	80	0	Killed after a dose of 15 gr. 18 hours previously.
8	Monkey	Veronal	„	5	10 „	50	0	Killed while in somnolent condition. Showed ataxia after fourth dose.
9	Monkey	Veronal	„	5	7 „	100	0	Had recovered activity and health when killed.
10	Monkey	Urethane	Subcutaneous injection	5 (1 c.c.)	12 „	60	0	Developed ataxia after 10 injections, and slept for 4 hours after twelfth injection. Died during night after this injection.
11	Monkey	Veronal	Subcutaneous injection	4 (1 c.c.)	10 „	40	—	Ataxia after 10 injections; killed 4 hours after fourteenth injection.
Y	Monkey	No drug given	—	—	—	—	—	Suffered from dysentery Shiga; the bacillus was isolated from intestine after death.

cerebellum of cats which had been fed with veronal, sulphonal, trional, luminal, dial and soneryl, and of monkeys which had received sulphonal, veronal and urethane. It consisted of a homogeneous mucinoid material similar to, but not identical with, amyloid, and was especially abundant in the tissues of animals Nos. 4*a*, 4*b*, 4*c*, 4*d*, 4*f*, 6*a* and 7.

Distribution and Properties of the Mucinoid Deposit.

1. *General.*—The substance was found only in the central nervous system of the animals; it was very marked in those which had received prolonged treatment, and could be produced by any one of the hypnotic drugs mentioned

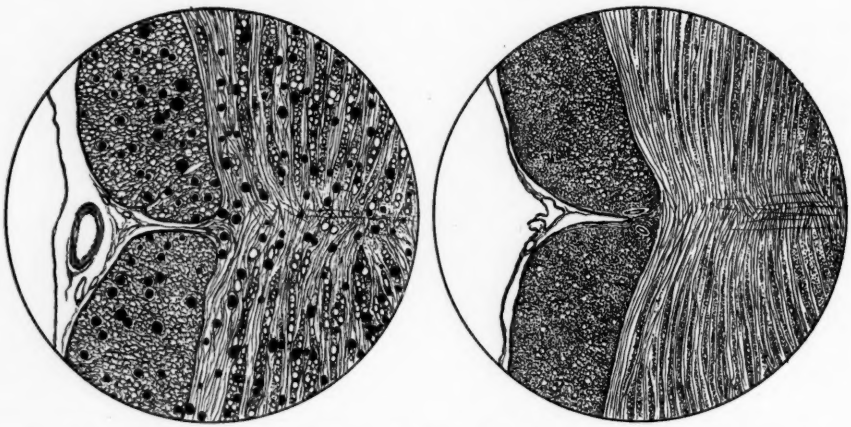
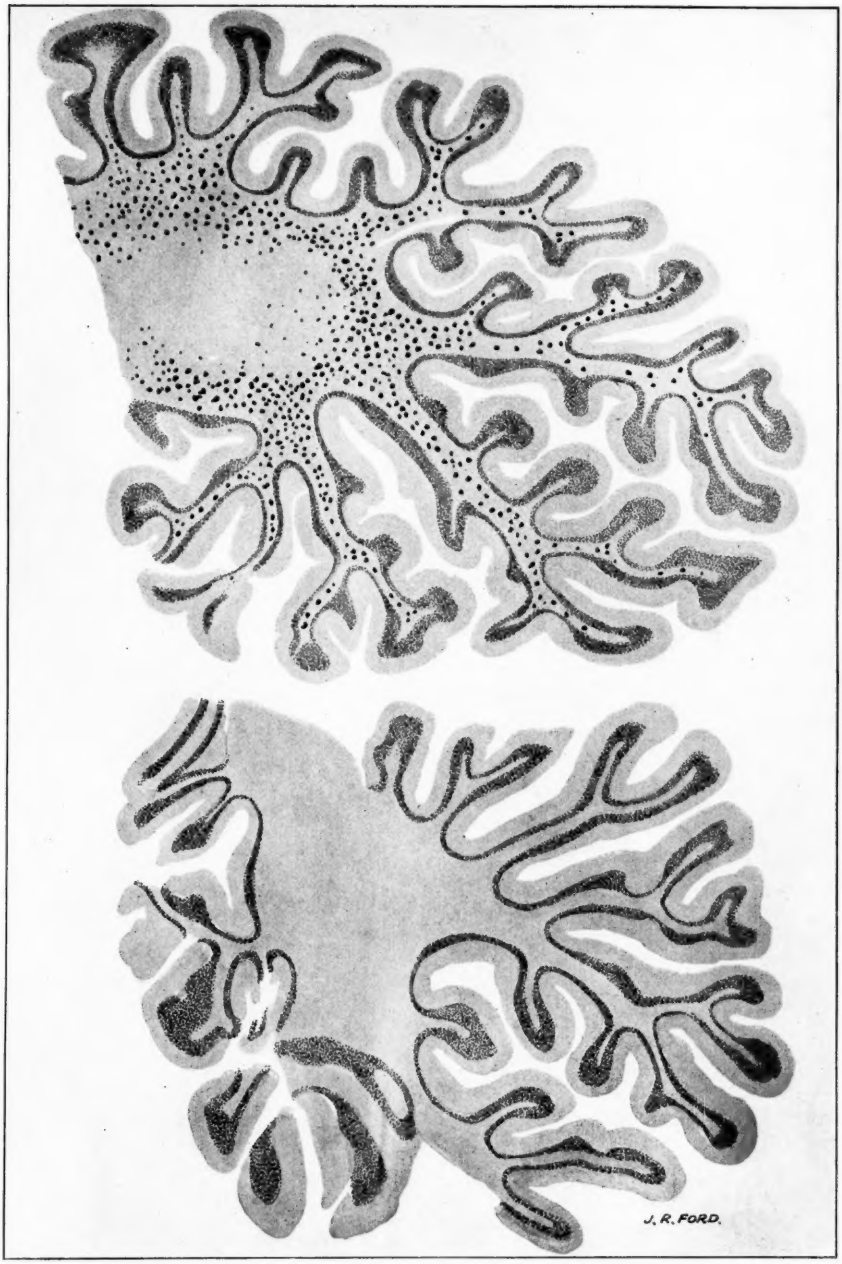


FIG. 1.—Sections of the pons of sulphonal-fed monkey (*L*) and normal (*R*), showing mucinoid material.

above. It was especially abundant in the white matter of the cerebellum (see plate), and occurred in quantity also in the white substance of the mid-brain, medulla and spinal cord (Figs. 1 and 2). To a lesser extent it was present in the grey matter of the cord, brain-stem, and in the cerebral cortex.

A systematic examination of the whole brain and cord of a cat (4*d*) which had received veronal for a long period showed a large quantity of this mucinoid substance through its central nervous system. It was especially abundant in the lumbar and cervical enlargements, where particles of large size up to 60μ were found. In the cervical part of the cord the main distribution formed a circular zone a short distance from the periphery of the cord, and many large globules occurred also within the grey matter. In the lumbar cord a zone of the material occurred at the periphery, whilst in the dorsal portions of the cord the distribution throughout the white matter was fairly even.

No trace of the mucinoid material could be detected in the viscera of the cats or monkeys, nor did the cerebro-spinal fluid, the sympathetic ganglia, the



Cerebellum of veronal-fed monkey, with normal control, showing mucinoid material limited to the white matter.

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choroid plexus or the pituitary body show the presence of any of this substance. The mucinoid material occurred in the form of discrete globules visible in unstained formalin-fixed paraffin sections as refractive homogeneous nodules varying from 5 to 60μ in diameter.

In some cases there was also a considerable amount of finer material having similar staining reactions. It often occurred near the blood-vessels, and appeared in spaces just outside the arteries and veins, apparently within the lymphatic or cerebro-spinal drainage system. Particles of the substance were found on the exterior of the cord and brain substance under the pia. Sections in which the fibres were specially stained showed these pushed aside by the mucinoid bodies, and in many cases the fibres were compressed together as they passed between masses of the substance.

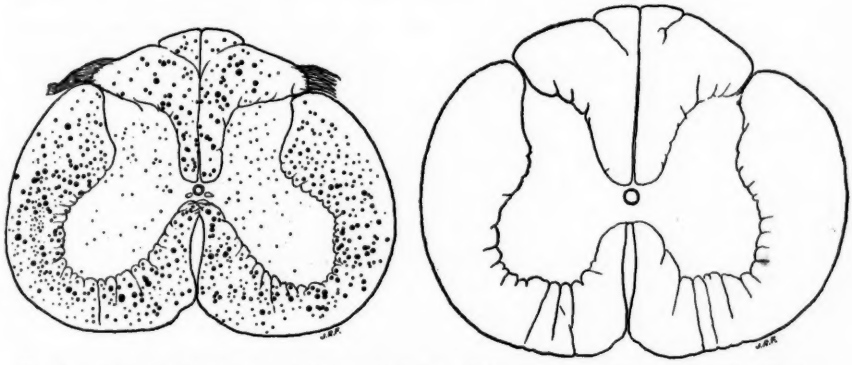

FIG. 2.—Sections of spinal cord of veronal-fed cat (*L*) and normal (*R*), showing distribution of mucinoid material.

The tissues of a number of healthy animals were examined for the presence of this material with negative results.

2. *Quantity.*—The amount of the mucinoid substance present in the spinal cord of monkey 6*b* was calculated by projecting the image of the stained section on to paper and plotting the area covered by the mucinoid substance. By weighing the paper representing the whole section and that corresponding to the mucinoid bodies, the quantity in a selected area of the cord was found to be 15 per cent. of the total area, and in one animal part of the white matter of the cerebellum in the region of the dentate nucleus showed large particles of this mucinoid substance amounting to 20 per cent. of the area as demonstrated by the above method; this percentage did not include the finer particles of the material. Cats which received only one dose of drug, though sufficient to produce a comatose condition within a few hours, showed no mucinoid material present (1*a*, 1*b*, 1*c*). Animals which had received 5 gr. of veronal (2, 9), for 4 or 5 days showed only small quantities in the white substance of the cerebellum, mid-brain and cord. The nervous tissues of the

animals which received similar quantities for 10 to 14 days all showed large quantities (3*a*, 3*b*, 3*c*, 5*d*, 8). The animals which were treated for still longer periods showed the mucinoid material in very large masses and in sufficient total quantity to give the white matter in the sections of tissues stained with thionin blue a decided reddish appearance (4*a*, 4*b*, 4*c*, 4*d*, 4*e*, 4*f*, 6*a*, 6*b*). It was found that animals which were allowed to recover for a number of days showed less mucinoid substance than similar animals which had been killed immediately after receiving doses of drug for the same period. Much smaller quantities were found in cats killed 5 to 10 days after discontinuing the drug (5*b*, 5*c*), and in the tissues of a cat killed 21 days after the last dose of veronal (5*a*) very fine particles only were found. This suggested that the product had been removed from the tissues.

3. *Properties of the mucinoid material: Physical.*—The globules of the formalin-fixed mucinoid substance were not dissolved by water, formol-saline, dilute acids, or dilute alkalies; they were insoluble in the organic solvents alcohol, ether, alcohol and ether, acetone, xylol or chloroform. They became swollen in sections under a cover-slip with warm water or warm salt solutions. They were collapsed by heat and by strong formaldehyde solution.

Chemical.—A number of histochemical tests were made on formalin-fixed tissue containing large amounts of mucinoid substance (frozen sections). It was found that the mucinoid material contained no free or masked iron, did not reduce ordinary Fehling's solution, or Benedict's solution, and gave no colour-reaction more than the general tissues when tested for the presence of phosphorus. The xantho-proteic reaction was also negative. Iodine solution stained the globules only a light yellow, which even appeared lighter than the surrounding tissue, proving absence of glycogen and differentiating the substance from amyloid.

Lugol's iodine solution followed by the application of sulphuric acid (with which amyloid produces a red colour changing to blue) gave no colour at all.

The material did not stain with acid fuchsin or carmine. Alum hæmatoxylin showed a definite affinity for the mucinoid material which gave a distinctly purple tint; Heidenhain's iron-hæmatoxylin gave a blue colour.

With Bismarck-brown in aqueous or carbolic solution the substance was stained an intense orange brown. The reaction was best obtained with paraffin sections, and the material was shown more distinctly by first treating sections with alizarin red and mordanting with ferric chloride solution. This double stain showed the mucinoid as yellow brown with the general tissue a deep purple. The results were not quite so good with frozen or celloidin sections but had the advantage of giving permanent specimens.

The substance showed metachromatic staining with toluidine blue, thionin blue, methyl violet and methylene blue, the mucinoid bodies taking a reddish tint, whilst the cells and fibres were stained blue.

With paraffin and celloidin sections thionin blue in 5 % carbolic acid gave very good staining, mordanting with mercuric salts as for mucin being quite unnecessary. When toluidine blue was used it was found that a short immersion in 0·1% solution of acetic acid effected an easy differentiation, and sections were best examined using a yellow screen, *e.g.* "Wratten G," which caused the bodies to appear deep red, having a greenish-blue background.

In all cases the action of alcohols, aniline oil, clove and cedar-wood oils was deleterious to the permanency of the stain. Sections were best dehydrated by carefully removing excess moisture by blotting and then treating with acetone or bergamot oil. So far it has been found that the colours in celloidin sections cannot be satisfactorily preserved, even mounting in resin or neutralized balsam not being successful in preserving the tints.

4. *Mucinoid material within nerve-cells.*—It is of special importance to note that in several instances a number of globules of this mucinoid substance was observed within the nerve-cells themselves, especially in the anterior horn-cells of the spinal cord and in the cells of the dentate nucleus of the cerebellum. We have spent a considerable amount of time in satisfying ourselves that the material was actually in the plane of the nucleus or nucleolus, and that it had not been introduced by mechanical agencies, *e. g.* the section knife. In a large number of specimens the remains of disintegrating cells associated with large masses of mucinoid material and having phagocytic cells in close proximity were observed.

Small quantities of the substance have also been noticed lying between and adjacent to the Purkinjë cells of the cerebellum.

Degenerative Changes in the Nerve-Cells.

The Purkinjë cells and the large cells of the dentate nucleus of the cerebellum, the horn-cells of the spinal cord and the Betz cells of the motor cortex were specially examined for signs of degeneration following drug treatment.

The specimens examined were fixed and prepared together with normal specimens, and were mounted, cut and stained together so that they should receive identical treatment throughout.

In animals which received only small doses of drugs, *e. g.* 1*a*, 1*b*, no appreciable changes were observed, but with examples in which a large amount was administered *e. g.* 4*a*, 4*b*, 6*a*, 6*b*, a very marked degree of alteration was observed in the tigroid bodies as stained by Nissl's method. This was especially marked in the Purkinjë cells and in the large cells of the mid-brain. In the case of tissues from 6*a* treated with sulphonal, the Purkinjë cells stained very diffusely compared with those of the normal specimens, Nissl granules in most cells being quite absent, and, where they existed, were very small and irregularly disposed. The cells were often very irregular in shape, the nucleus was enlarged, and there was a marked tendency to assume an eosinophile reaction (Fig. 3).

In the medulla and pons chromatolysis with enlargement of nucleus was observed, some cells showing vacuolation and extrusion of the nucleus, and the presence of mucinoid substance was frequently observed adjacent to these disintegrating cells.

The specimens from 6*b* and 4*a* similarly showed this condition. The anterior horn-cells of the cord of cat 3*b*, which received 10 doses of veronal, were found to be distinctly affected by the drug treatment; as compared with the normal they stained more diffusely by polychrome methylene-blue and the Nissl bodies were smaller and fewer. In many cases the cells were misshapen with eccentric nuclei. Vacuolation was noted, and many phagocytic cells were seen close to the degenerating cells.

Examination of Fibres of Nervous Tissues.

The spinal cord, mid-brain and cerebellum of the animals were carefully examined for degenerated fibre-tracts by the Marchi and Kulschitsky-Pal methods, but none were found in any case. The entire mid-brain and cord of cat 4*d* examined by these methods showed no fibre-tract degeneration. The fine fibrils in all cases showed good staining with the Del Rio Hortega method.

Occurrence of the Mucinoid Substance in Other Conditions.

Small amounts of the substance with similar staining reactions have been found in the formalin-fixed tissues of animals which had not received drugs,

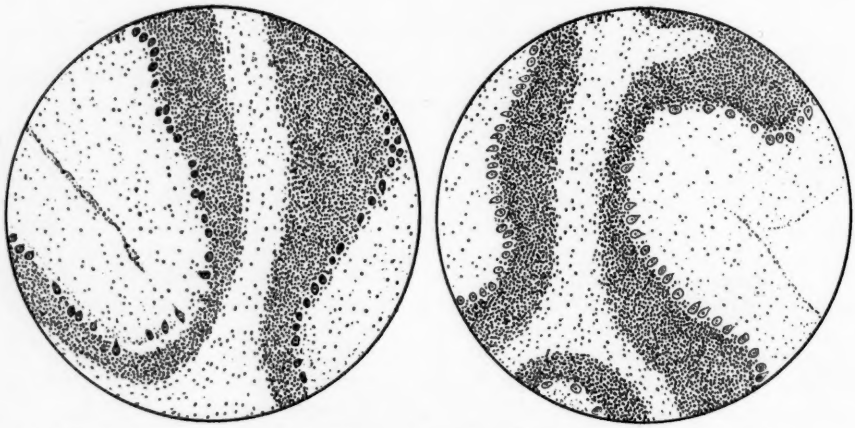

FIG. 3.—Sections of cerebellum of sulphonal-fed monkey (*R*) and normal (*L*), showing absence of Nissl granules in the Purkinjë cells.

but which had suffered from chronic illness (*X*, *Y*). There was, however, a marked difference in the sections from these animals, the mucinoid particles being much smaller, and occurring chiefly as fine dust as distinct from the large masses found in the drug-treated animals. The monkey (*Y*) dead from dysentery showed the material chiefly in the cerebellum and cord, and the cat (*X*) killed after a long period of gastro-intestinal catarrh and diarrhœa had the material distributed more uniformly in the form of a fine dust throughout the nervous system. A case of human myxœdema, in whom post-mortem examination of the thyroid-fibrous remnant showed complete absence of thyroid epithelium and chemical examination total absence of iodine, showed some similar substance lying in the perivascular and lymphatic drainage system in the medulla and cerebellum.

Buscaino (1921) has described the appearance of "uniform clusters of disintegration areas giving a reddish violet stain with toluidine azur," in

the nervous system of cases of dementia præcox; but it is not apparent from the paper whether the patients had received any treatment with hypnotic drugs.

The same author has found similar areas in dogs which had been injected with formic acid. In this connection we examined a rabbit injected on 3 days with 10 per cent. formic acid as described by him for dogs, and confirmed the presence of a small amount of mucinoid material similar to that produced by the hypnotic drugs, but in much less quantity, the small particles found being similar to the condition in the animals suffering from chronic illness.

DISCUSSION.

The occurrence of material in such remarkable amounts in the nervous system of the treated animals is the most interesting feature of the effects of continued treatment with hypnotic drugs.

Although the drugs sulphonal and veronal have been isolated from brain-tissue (Russel and Parker, 1914), and would be present in the nervous systems of animals treated as above; the mucinoid substance does not consist of these, since it shows entirely different physical properties from these compounds. It also appears improbable that substances of such widely different chemical constitutions as the barbitone and sulphonal groups of drugs would enter into an important composition of the mucinoid material.

So far we have not been able to demonstrate the substance in living tissues, and it is only after formalin fixation that it appears in the characteristic form described above.

As shown above, the mucinoid substance occurs sometimes actually within the nerve-cells; it is therefore probable that it is a metabolic product of the nerve-cells themselves which have been damaged by the drugs; also many cells have the Nissl substance much diminished, whilst others are disintegrated completely, leaving only an outline with masses of mucinoid lying adjacent to them, and phagocytic glia-cells apparently digesting the remnants. Possibly the mucinoid substance is itself a product of the ordinary metabolism of the neurones, which normally is quickly removed and thus never accumulates into large masses. Under the action of the drugs, however, either much larger quantities of the material are produced, or it is not removed so readily, therefore accumulating within the cells and adjacent to them, and in time aggregating into large masses filling the perivascular and lymph-spaces in the neighbourhood.

The experiments conducted show that it disappears on ceasing administration of the drugs and the animals soon regain their activity and intelligence.

The permanence of the damage done is, however, difficult to estimate, since histologically we have shown that many nerve-cells are damaged beyond hope of recovery.

Scharlach R and other fat lipoid stains do not stain the substance in formalin-fixed sections, consequently the material described above shows differences from the lipoid excretions of Orr and Sturroch (1922), Buscaino (1914) and other workers.

It is submitted that incidental to the demonstration of this material in formalin-fixed sections in drug-treated animals, the presence or absence of this

material suggests further research into the metabolism of the neurones, and we are continuing investigations upon these lines.

SUMMARY.

1. In all cases where hypnotic drugs are administered over a period of 7 or more days, numerous masses of a peculiar mucinoid material 5 μ to 60 μ in diameter are found distributed throughout the central nervous system. Normal control tissue gives complete absence of this material. The fixed material in its staining reactions shows properties somewhat similar to amyloid.

2. Chromatolysis, loss of Nissl substance and signs of cell degeneration are present in the nerve-cells of the cerebellum, mid-brain and spinal cord after intensive treatment with any of the hypnotic drugs. The cell degeneration is accompanied by the appearance of numbers of phagocytic cells, which appear to digest the nerve-cells.

The above work has been done in the laboratories of the Joint Board of Research for Mental Diseases, City and University of Birmingham, and we have to thank the Medical Research Council for the initial cost of the monkeys. Thanks are also due to Sir William Willcox, M.D., K.C.I.E., C.B., C.M.G., for valuable suggestions and help.

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